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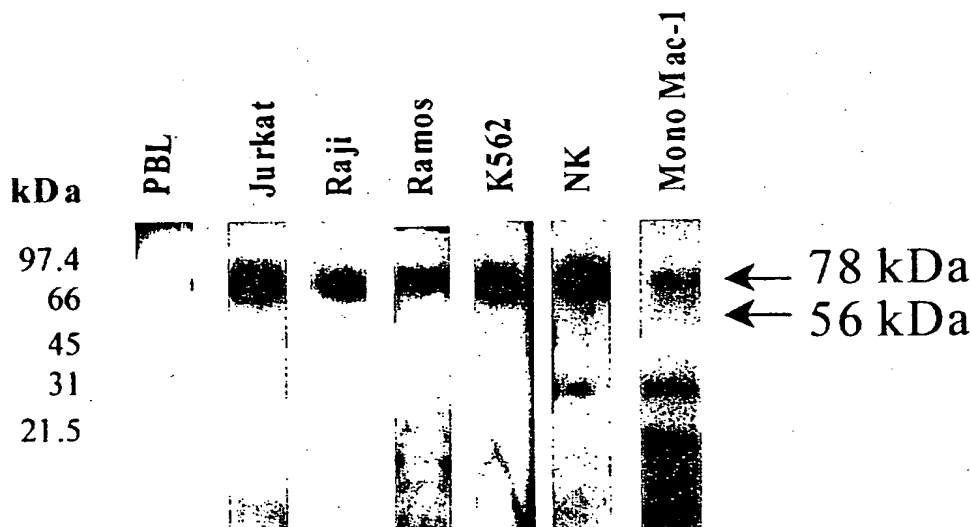
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(54) Title: METHODS FOR SCREENING FOR A COMPOUND USEFUL IN THE TREATMENT OR PREVENTION OF LYMPHOCYTIC DISORDERS, FOR INHIBITING LYMPHOCYTE ACTIVITY AND PREVENTING OR TREATING LYMPHOCYTIC DISORDERS



(57) Abstract: A method of screening for a compound useful in the treatment of a disease or condition characterized by an immune cells disorder, wherein said cell expresses NTPDases, said method comprising the steps of contacting a candidate compound with ecto-nucleoside triphosphate diphosphohydrolase (NTPDase), wherein the candidate compound is selected if the activity of said NTPDase is reduced in the presence of the candidate compound as compared to that in the absence thereof. A method for inhibiting an immune cell activity in a mammal, comprising targeting immune cells with an effective amount of a NTPDase inhibitor. A method to prevent or reduce the risk of rejection of transplanted tissue or organ, comprising administering to said animal an effective amount of NTPDase inhibitor.

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TITLE OF THE INVENTION

METHODS FOR SCREENING FOR A COMPOUND USEFUL IN THE TREATMENT OR PREVENTION OF LYMPHOCYTIC DISORDERS, FOR INHIBITING LYMPHOCYTE ACTIVITY AND PREVENTING OR TREATING LYMPHOCYTIC DISORDERS

FIELD OF THE INVENTION

[0001] The present invention relates to methods for screening for a compound useful in the treatment or prevention of lymphocytic disorders, for inhibiting lymphocyte activity and preventing or treating lymphocytic disorders. More specifically, the present invention is concerned with NTPDase inhibitors and methods of using same.

BACKGROUND OF THE INVENTION

[0002] Many conditions and disease involve defective or undesirable T lymphocyte proliferation or activation; these disease and conditions include graft-host reaction such as organ or tissue rejection, neoplasia, tumors, T cell leukemia, immune diseases and various diseases and conditions in oncology and oncohematology, etc.

[0003] In particular, the T cell leukemia is a T lymphocyte disorder: a progressive or aggressive disease. The T cell malignancies can be classified into two principal groups: 1-T cell acute lymphoblastic leukemia (ATLL) and 2-T cell chronic lymphoblastic leukemia.

[0004] Actual clinical therapies include chemotherapy, radiotherapy, bone marrow transplantation, or a combination of this treatment. Patients are almost always resistant to chemotherapeutic agent and were refractory to other

therapies. ATLL is a very aggressive T cell malignancy for which no successful treatment is yet available. The acute forms have a poor outcome with overall survival ranging from 5 to 13 months. ATLL does not respond or only transiently to combination chemotherapy.

[0005] Patients with chronic forms become refractory to therapy. The poor response to therapy related to chemotherapy resistance. Results are not superior with immunotherapy.

[0006] Adenosine triphosphate (ATP) and Adenosine diphosphate (ADP) act as signaling molecules for cells of virtually all origins. Indeed these purines, which are released from the cells by exocytotic and non-exocytotic mechanisms can interact with specific receptors and thereby can influence the different physiological systems. One finds on the cell surface two classes of receptors which respond to ATP and ADP and identified as purinoceptors, and one additional class of purinoceptors which respond to adenosine. In the immune system, the role of purines is still poorly understood. It is well known however that extracellular ATP can modulate certain responses of various lymphocyte cell populations such as DNA synthesis, blastogenesis and mediated cell killing via specific cell membrane purinoceptors. In these respects, it has been shown that ATP inhibits natural killer cell activity (11) of human and murine origins and phagocytosis in mouse macrophages (3). Extracellular ATP can stimulate *in vivo* DNA synthesis of thymocytes but inhibits DNA synthesis of spleen and peripheral blood lymphocytes (6). Baricordi et al (7), reported that AT had a synergistic effect on DNA synthesis stimulated by selective T-cell mitogens such as PHA or anti-CD₃ monoclonal antibody.

[0007] At least two functionally distinct purinoceptors subtypes have

been described in lymphocytes: 1) that coupled to G proteins, namely the P2Y receptor linked to IP3 generation and Ca²⁺ mobilization from intracellular stores and 2) the P2X/P2Z receptor which gates a channel permeable to Na⁺ and Ca²⁺ (7). A P2Y receptor has been described in human B lymphocytes and has been reported to be absent from T cells (13-17). Whereas nucleotide activated ion channel has been shown to be expressed to a low level in normal B-lymphocytes and to be upregulated in resting mouse T and B-lymphocytes, leukemic peripheral blood lymphocytes. According to Baricordi et al. (7), human peripheral blood lymphocytes and purified T lymphocytes express a P2 X7 purinoceptor and ionic channel gated by extracellular ATP that is involved in the control of mitogenic stimulation by different stimuli. The extracellular concentration of the agonist (ATP, ADP or adenosine) which elicits the cellular response is determined by several parameters: i) rate of release and diffusion, ii) metabolism by ectonucleotidases and iii) binding affinity of the receptors. In this context, the role played by ectonucleotidases, and more specifically ectonucleoside triphosphate diphosphorylase (NTPDase), appears to be determinant. In this respect, immuno competent cells, such as lymphocytes and macrophages, express ectonucleotidases activities. Ectonucleotidase which catalyses the hydrolysis of ATP to ADP has been reported to B cells (18), macrophages (20), natural killer (NK) cells and CTL (21). It has been proposed that an ecto ATPase could protect murine CTL cells from the lytic effects of extracellular ATP released during granules exocytosis (1, 10, 22) and that this ecto ATPase was required for the cytolytic activity of NK cells. Recent data on ATPDase show that NTPDase 1 (CD₃₉), which is distributed on the cell surface of many cell types, plays a key role in the conversion of extracellular ATP to ADP and ADP to AMP. This enzyme, put in evidence many years ago by Lebel et al. (36) in pig pancreas, has been recently identified in primary and secondary lymphoid organs including spleen, thymus, tonsils, and Peyer's patches and isolated lymphocytes and macrophages from pig spleen

(Benrezzak et al. (1999)). It has also been demonstrated that CD₃₉, a lymphoid cell activation antigen (30), corresponds to human NTPDase (31). Kansas et al. (32) cloned the latter molecule and studied its distribution and they reported that this protein is expressed on activated NK, B, and T cells of peripheral blood and is found in certain lymphoid tissues, namely tonsils and thymus. Despite these reports demonstrating the presence of NTPDase (CD₃₉) in the immune system, the physiological role played by the NTPDase in the immune response remains to be clarified. A relevant obstacle to the understanding of the NTPDase functions is a lack of specific inhibitors, i.e. an inhibitor that does not interfere with purinoceptors. A recently described NTPDase inhibitor, BGO 136, a naphthyl derivative also known as 1-hydroxynaphthalene-3,6 disulfonic acid, produced a mixed type of inhibition with K_i s of 380 μ M with ATP and ADP as substrate, respectively (Gendron et al. 2001). Biochemical and pharmacological characterisations of a further NTPDase inhibitor, the 8 Bus ATP, are described in Gendron et al. (2000), J Med Chem. The US patent application no. 09/591,177 in the name Beaudoin *et al.* teaches that various others compounds are also able to inhibit NTPDase activity.

[0008] There remains a need to determine the activity and functions of the NTPDase. In particular, there remains a need to determine whether NTPDase has a direct implication in immune responses.

[0009] There also remains a need to provide new means of preventing or treating diseases or conditions involving defective or undesirable T lymphocyte proliferation or activation.

SUMMARY OF THE INVENTION

[0010] The present invention teaches that NTPDase is directly involved

in the immune response. It teaches that NTPDase is an agonist of T lymphocyte proliferation and humoral response. It also teaches that NTPDase inhibitors are able to inhibit T lymphocyte proliferation and humoral response. More specifically, it teaches that NTPDase inhibitors may be used to prevent graft rejection.

[0011] More specifically, the present invention also teaches the inhibitory effects of BGO 136, erythrosin B, 8 Bus-ATP, 8 Bus-AMP on cell specific mitogenic stimulation and lymphoproliferative properties of T cells and on primary antibody response to a T-cell dependent antigen. These findings emphasize the importance of NTPDase for the T-helper cell for functions in humoral responses induced by T cell dependent antigens.

[0012] Results obtained on mouse revealed efficacy of NTPDase inhibitors and tolerance for long-term treatment.

[0013] The present invention provides uses and methods of using NTPDase inhibitors in the prevention or treatment of disease or condition characterized by a T lymphocytic disorder including graft vs host reaction following organ or tissue transplant, blood disorders such as neoplasia (leukemia and lymphoma), autoimmune disorders such as rheumatoid arthritis and psoriasis, and various other oncological and oncohematological diseases and conditions.

[0014] Composition within the scope of the present invention should contain the NTPDase inhibitor in an amount effective to achieve the desired therapeutic effect while avoiding adverse side effects. The dosage will be adapted by the clinician in accordance with conventional factors such as the extent of the disease and different parameters from the patient. Typically,

0.001 to 50 mg/kg/day of the NTPDase inhibitor in accordance with the present invention will be administered to the mammal. Pharmaceutically acceptable preparations and salts of the NTPDase inhibitor are within the scope of the present invention and are well known in the art (Remington's Pharmaceutical Science, 16th Ed., Mack Ed.).

Assays to identify NTPDase of the present invention

[0015] Preferred methods for testing the ability of candidate compounds to inhibit the NTPDase activity are presented herein. It will be understood that the invention is not so limited. Indeed, often assays well known in the art can be used in order to identify non-competitive, extracellular antagonists of the present invention.

[0016] It shall be understood that the "*in vivo*" experimental model can also be used to carry out an "*in vitro*" assay.

In vitro assays

[0017] In a preferred embodiment, candidate inhibitors are tested for their ability to inhibit NTPDase ability to modulate lymphocytic proliferation with the incorporated triated thymidine method. Other ways of determine cellular proliferation may be use. For instance cell count and the assays described in Baker F.L. et al. (1995) Cell Prolif. 28(1):1-15; Cheviron N. et al. (1996) Cell Prolif. 29(8):437-46; Hu Z. W. et al. (1999) J: Pharmacol. Exp. Ther. 290(1):28-37; and Elliott K. et al. (1999) Oncogene 18(24):3564-73.

In vivo assays

[0018] The assays described above may be used as initial or primary screens to detect promising lead compounds for further development. Lead inhibitors will be further assessed in additional, different screens. Therefore,

this invention also includes secondary screens which may involve various assays utilizing mammalian cell lines expressing these purinoceptors or other assays.

[0019] In yet other preferred embodiments, candidate inhibitors are tested for their ability to inhibit NTPDase's ability to elicit humoral response and specifically antibody production. Any methods known in the art including those described herein for determining antibody production may be used including the techniques involving the formation of hemolytic plaques, etc.

[0020] Tertiary screens may involve the study of the identified inhibitors in animal models for clinical symptoms. Accordingly, it is within the scope of this invention to further use an inhibitor identified as described herein in an appropriate animal model such as a rat or a mouse. For example, a inhibitor can be used in an animal model to determine the efficacy, toxicity, or side effects of treatment with such an agent. Alternatively, an inhibitor identified as described herein can be used in an animal model to determine the mechanism of action of such an agent. Furthermore, this invention pertains to uses of novel agents identified by the above-described screening assays for treatment (e.g. treatments of different types of disorders associated with a deregulated, defective, undesirable immune cells activity), as described herein..

[0021] More specifically, in accordance with the present invention, there is provided a method of screening for a compound useful in the treatment of a disease or condition characterized by an immune cells disorder, wherein said cell expresses NTPDases, said method comprising the steps of contacting a candidate compound with ecto-nucleoside triphosphate diphosphohydrolase (NTPDase), wherein the candidate compound is selected if the activity of said NTPDase is reduced in the presence of the candidate compound as compared to that in the

absence thereof. In a specific embodiment, the contacting of the candidate compound with the NTPDase is performed in an immune cell selected from the group consisting in normal T lymphocyte, normal B lymphocyte, normal NK cell, normal macrophage, normal monocyte, Jurkat cell, Raji cell, Ramos cell, MonoMac™ cell, K562 cell and U937 cell.

[0022] According to a further aspect of the present invention, there is provided a method for inhibiting an immune cell activity in a mammal, comprising targeting immune cells with an effective amount of a NTPDase inhibitor. In specific embodiments, the method is further characterized by one or more of the following characteristics: the immune cells are normal lymphocytes (such as T and B lymphocytes) or tumoral immune cells (such as T or B neoplastic lymphocytes, and more specifically Jurkat cells), the activity is the T lymphocyte proliferation or the production of antibodies. In more specific embodiments, the activity is induced either by organ or tissue transplant, by an allergen, or by autoimmune diseases. In further specific embodiments, the NTPDase inhibitor is selected from the group consisting of BGO 136, erythrosin B, nucleotide or nucleotide derivative including AMP, 8 Bus-AMP and 8 Bus-ATP, and analogues thereof.

[0023] In a further aspect of the present invention, there is provided a method to prevent or reduce the risk of rejection of transplanted tissue or organ, comprising administering to the mammal an effective amount of NTPDase inhibitor. In a specific embodiment, the NTPDase inhibitor is selected from the group consisting of BGO 136, erythrosin B, nucleotide or nucleotide derivative including AMP, 8 Bus-AMP and 8 Bus-ATP, and analogues thereof.

[0024] In a further aspect of the present invention, there is provided a composition for use as an immunosuppressive agent in graft transplant comprising an effective amount of BGO 136 or BGO 136 analogue in a pharmaceutically acceptable carrier.

[0025] In a further aspect of the present invention, there is provided a use of a BGO 136 or BGO 136 analogue in the making of a medicament for use as an immunosuppressive agent in graft transplant.

[0026] NTPDase is a family of enzymes which catalyse the sequential hydrolysis of the gamma and betaphosphate bonds of nucleoside tri and diphosphates and characterized by homologous sequences (apyrase conserved regions). As used herein, the term "NTPDase" refers to any member of this family.

[0027] As used herein, the term "disease or condition characterized by an immune cell disorder" is meant to include disease and conditions characterized by defective or undesirable immune cell activity. It includes normal T lymphocytes proliferation and normal B lymphocytes humoral response, any dysfunction in normal immune cells including monocytes, NK cells, and lymphocytes, neoplastic immune cells proliferation, etc. Without limiting the generality of the foregoing, such diseases and conditions include graft vs. host reaction following organ or tissue transplant (including graft rejection), blood disorders such as neoplasia (leukemia and lymphoma), autoimmune disorders including rheumatoid arthritis, psoriasis, Crohn disease and certain diabetes, oncological and oncohematological diseases and conditions.

[0028] As used, the terms "neoplastic", and "tumoral" are used interchangeably.

[0029] As used herein, the term "NTPDase activity" is meant to include the enzyme's ability to hydrolyse ATP and ADP and any downstream effect of this enzymatic reaction including lymphocyte proliferation and humoral response.

[0030] As used herein, the term "lymphocytes" is meant to include normal lymphocytes and neoplastic/tumoral lymphocytes. Without limiting the generality of the foregoing, this term is meant to include normal T and B lymphocytes such as those found in normal mammals, and neoplastic/tumoral T and B lymphocytes such as Jurkat, Rami and Ramos cells.

[0031] As used herein, the term "effective amount" is meant to refer to an amount of NTPDase inhibitor administered in a single dose or in multiple doses sufficient to reduce or abrogate the undesirable lymphocyte activity in the cell or the animal to which it is administered while avoiding adverse side effects. Without limiting the generality of the foregoing, the results presented herein demonstrate that an amount of 4,3mg/g of base weight of transplanted spleen is sufficient to reduce the spleen increase by 56%. It refers to the amount necessary to produce a benefit to the cell or animal to which it is administered as determined by a person of ordinary skill in the art.

[0032] As used herein, the term "BGO 136 analogue" is meant to refer to any naphtyl derivative having the ability to inhibit an NTPDase activity. Furthermore, the F. B. Gendron doctorate thesis submitted to the University of Sherbrooke on inhibitors defines the conformational characteristics that enable an analogue to possess inhibitory abilities.

[0033] As used herein, the term "AMP analogue" is meant to refer to any adenosine 5'-monophosphate derivative having the ability to inhibit an NTPDase activity including 8-BUS AMP. Since AMP is the last member of the reaction, it is submitted that it is reasonably predictable that any AMP analogue will inhibit NTPdase.

[0034] As used herein, the term "ATP analogue" is meant to refer to any adenosine 5'-triphosphate derivative having the ability to inhibit an NTPDase

activity including 8-BUS ATP. Gendron *et al.* (2000a) and Nahum *et al.* (2002) describe such ATP analogues.

[0035] As used herein, the term "erythrosin B" is meant to refer to any member of the erythrosin family or erythrosin derivative having the ability to inhibit an NTPDase activity.

[0036] As used herein, the term "nucleotide derivative" is meant to refer to any nucleotide having been subjected to any modification preserving its ability to inhibit an NTPDase activity. Assays as described in Picher *et al.* (1996) showed that modifications on the ATP phosphate chain prevent ATP hydrolysis consequently granting resulting molecules with inhibitory activities.

[0037] As used herein, the term "humoral response" is meant to include antibody production.

[0038] Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of preferred embodiments thereof, given by way of example only with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0039] In the appended drawings:

[0040] Figure 1 shows the presence (the 78KDa and 56KDa lines) of NTPDase in human lymphoid cells. In this figure, PBL: normal peripheral blood cells; Ramos : B leukemia cells; Jurkat : T leukemia cells; K562 : erytroleukemia cells; Raji : B leukemia cells; NK: natural killer cells;

Monomac™ : monocytes leukemia cells;

[0041] Figure 2 shows the effect of BGO 136 on NTPDases activity of human peripheral blood lymphocytes (PBL). Panels A and B show BGO 136's effect on the ATPDase and ADPDase functions, respectively. Eight (8) assays were performed each in triplicate. NTPDase 1 and 2 (in a lesser extent) were used;

[0042] Figure 3 shows the inhibitory effect of BGO 136 on human peripheral blood lymphocytes (PBL) proliferation. Panel A presents results with a control (unstimulated cells); Panel B presents results with cells stimulated by ConA™ and Panel C presents results with cells stimulated with LPS™. Three (3) experiments were performed each in quadruplicate. [inhibitor] : indicates inhibitor concentration;

[0043] Figure 4 shows the correlation between NTPDase activity of cells subjected to BGO 136 as presented in Figure 2 and proliferation assay of peripheral blood lymphocytes (PBL) subjected to BGO 136 as presented in figure 3. Each set of point corresponds to a single concentration of the inhibitor.(concentration used are 0, 1, 5 and 10 mM of BG0136);

[0044] Figure 5 shows the effect of BGO136 on humoral response (antibodies production) 21 days after a first injection (Panel A), 32 days after first injection (Panel B); and 40 days after a first injection (Panel C). In this figure, DM : indicates that multiple doses of BGO136 were given; +Ag : mice received BSA as Ag T cell dependent; - Ag : mice received only vehicle; BGO136 was given in a single dose at the mentioned concentration (400 or 800 mM). Each group contained ten (10) mice;

[0045] Figure 6 shows the effect of erythrosin B on NTPDase activity of human peripheral blood lymphocytes (PBL). Six (6) experiments were performed each in triplicate;

[0046] Figure 7 shows the effect of erythrosin B on human peripheral blood lymphocytes (PBL) proliferation. Three (3) experiments were performed each in sextuplicate;

[0047] Figure 8 shows the correlation between NTPDase activity of cells subjected to erythrosin B as presented in figure 6 and proliferation assay of peripheral blood lymphocytes (PBL) subjected to erythrosin B as presented in figure 7. Each set of point corresponds to a single concentration of the inhibitor. Concentrations used are 0, 10, 30, 50 and 100 μ M of erythrosin B;

[0048] Figure 9 shows the effect of erythrosin B on humoral response (antibody production) 21 days after first injection (Panel A), 32 days after first injection (Panel B) and 40 days after first injection (Panel C). In this figure, DM : Multiple doses of Erythrosin B were given; Groupe Ag : mice received only BSA as Ag T cell dependant without inhibitor; Erythrosin B was given in a single dose as mentioned 0.25g/kg or 0.5g/kg. Each group contain 10 mice;

[0049] Figure 10 shows the inhibitory effect of 8-BuS ATP on NTPDase activity of human peripheral blood lymphocyte. One (1) experiment was performed in duplicate;

[0050] Figure 11 shows the inhibitory effect of 8-BuS-ATP on the human peripheral blood lymphocytes (PBL) proliferation. Three (3) experiments were performed each in sextuplicate;

[0051] Figure 12 shows the correlation between NTPDase activity of cells subjected to 8-BuS-ATP as presented in figure 10 and proliferation assay of peripheral blood lymphocytes (PBL) subjected to 8-BuS-ATP as presented in Figure 11. Each set of point corresponds to a single concentration of the inhibitor. Concentrations used are 0, 10, 50 and 100 μ M of 8-BuS-ATP;

[0052] Figure 13 shows the inhibitory effect of 8-BuS AMP on NTPDase activity of human peripheral blood lymphocyte. Two (2) experiments were performed each in triplicate;

[0053] Figure 14 shows the inhibitory effect of 8-BuS-AMP on the human peripheral blood lymphocytes (PBL) proliferation. Three (3) experiments were performed each in sextuplicate;

[0054] Figure 15 shows the correlation between NTPDase activity of cells subjected to 8-BuS-AMP as presented in figure 13 and proliferation assay of peripheral blood lymphocytes (PBL) subjected to 8-BuS-AMP as presented in figure 14. Each set of point corresponds to a single concentration of the inhibitor. Concentrations used are 0, 10, 30 and 100 μ M of 8-BuS-AMP;

[0055] Figure 16 shows the inhibitory effect of beta-gamma methylene ATP on NTPDase activity of human peripheral blood lymphocytes (PBL). Two (2) experiments were performed each in triplicate;

[0056] Figure 17 shows the inhibitory effect of beta-gamma methylene ATP on the human peripheral blood lymphocytes (PBL) proliferation. Three (3) experiments were performed each in sextuplicate;

[0057] Figure 18 shows the correlation between NTPDase activity of cells subjected to beta-gamma methylene ATP as presented in figure 16 and proliferation assay of peripheral blood lymphocytes (PBL) subjected to beta-gamma methylene ATP as presented in figure 17. Each set of point corresponds to a single concentration of the inhibitor. Concentrations used are 0, 50, 100 and 250 uM of beta-gamma Me-ATP ; and

[0058] Figure 19 shows the inhibitory effect of NTPDase inhibition on neoplastic T cell growth Results obtained with erythrosin B, BGO 136, AMP, beta-gamma methylene ATP, 8-BuS-ATP are shown in Panels A to E, respectively. For each inhibitor, two (2) experiments were performed each in quadruplicate.

DESCRIPTION OF SPECIFIC EMBODIMENTS

Isolation of human lymphocytes

[0059] Human lymphocytes were obtained from peripheral venous blood of normal and healthy medication-free volunteers. Fresh blood collected in EDTA glass tubes was layered into Histopaque™ – 1077 (a solution of Ficoll™ and sodium diatrizoate adjusted to a density of 1.077, Sigma, USA) and centrifuged at 400 g for exactly 30 min, at room temperature. During centrifugation, erythrocytes and granulocytes were aggregated by Ficoll™ and rapidly sedimented, whereas lymphocytes remained at the plasma interface. Plasma (the upper layer) was carefully removed to prevent disturbance of the buffy coat. The latter fraction rich in lymphocytes was recovered with a pasteur pipette and washed twice with RPMI™ 1640 medium by centrifugation, for 10 min, at 250 X g. The final pellet was suspended in fresh RPMI™ 1640 medium supplemented with 2mM L-glutamine, 10% foetal bovine serum inactivated at 56°C for 30 min, and antibiotics: penicillin 100 units/ml, streptomycin 100 ug/ml

and amphotericin 2.5 ug/ml. Cells were counted with the hemacytometer. Viability, tested with Trypan™ blue exclusion assay, was superior to 90%. The cell preparation named PBL (for peripheral blood lymphocyte) was immediately used for NTPDase assays and for mitogenic responses to stimulators.

Maintenance of lymphoblastoid human cell lines

[0060] Human cell lines were given by Dr. Jana Stankova (Department of Immunology, Université de Sherbrooke). Jurkat cells were derived from human T cell leukemia. Raji and Ramos cells are B-cell lymphomas derived from peripheral blood of patients with Burkitt lymphoma. MonoMac™-1 is a cell line derived from the peripheral blood of a 64 year-old male with active monocytic leukemia. K562, which is used as highly sensitive target for NK cell activity is an erythroleukemia cell line. U937 is a promonocytic myeloid cell and finally the Y2 T2 C2 (NY) cell is a human natural killer cell line. These lymphoblastoid cell lines which grow in suspension as single cells without attachment to glass, were maintained by passage in complete RPMI 1640 medium with 2 mM L-glutamine containing 10-20% heat inactivated foetal bovine serum [V/V] and antibiotics (100 units/ml of penicillin), streptomycin 100 ug/ml and 2.5 ug/ml of amphotericin) at 37°C in a humidified 5% CO₂/95% air atmosphere. Mono-Mac™ 1 cells were maintained in the same complete RPMI™ 1640 medium supplemented with 1 x non-essential amino acids and 1mM Na-pyruvate at 37°C with 5% CO₂.

Mitogen stimulation and proliferation assay

[0061] A thymidine uptake assay was used to evaluate the effect of NDPase inhibitors on the proliferative activity of normal lymphocytes.

[0062] Cell suspensions of fresh human peripheral blood lymphocytes (PBL) were resuspended in RPMI™ 1640 medium supplemented with 10%

foetal bovine serum at a concentration of 2×10^6 /ml. Aliquots of 100 µl were plated on a Falcon™ flask (96 – well) and stimulated with 10 µg/ml of (Con-A) or 40 µg/ml of (LPS™) at a final volume of 0.2 ml/well. ConA™ and LPS™ were used to stimulate preferentially T cells and B cells, respectively. Cell cultures \pm inhibitors were incubated for 48 h at 37°C in a 5% CO₂ atmosphere [³H] thymidine (1 µg/well, specific activity 5.0 ci/mmol) was added to each microplate in a volume of 50 µl. After an additional a 4 h incubation at 37°C, cells were collected with a cell harvester and ³H thymidine incorporation was measured in triplicate samples by liquid scintillation counting.

NTPDase assays

[0063] Prior to the assays, human normal lymphocytes as well as leukemic cell lines, were washed three times with phosphate-free saline in 95 mM NaCl, 1mM CaCl₂, 2mM MgCl₂ and 45mM Tris – HCl buffer (pH 7.4). Enzyme assays were carried out at 37°C in 1 ml of the following assay medium: 95mM NaCl, 5mM KCl, 1mM CaCl₂, 2mM MgCl₂, 5mM glucose, 0.05% BSA, 5mM tetramisole and 45mM Tris-HCl (pH 7.5). The reaction was started by adding the substrate (200µM of ATP or ADP) and stopped with 250 µl of the Malachite green reagent. Controls were run with the enzyme added after the Malachite green reagent. Where indicated, 10mM sodium azide (NaN₃) or 1mM, 5mM and 10 mM of BGO 136 were added directly to the assay mixture. Inorganic phosphate was estimated according to Baykov et al.⁽¹⁾ and enzyme activity was either expressed as nmoles of Pi released/min/mg of protein which corresponds to mUnits ⁽²⁾ or as nmoles Pi/min/10⁶ cells. Where indicated, lymphoid cells were lysed by three freeze thaw cycles in saline buffer and protein was measured with the method of Bradford using bovine serum albumin as a standard ⁽³⁾.

Electrophoresis and Western blotting

[0064] Lymphocytes from human peripheral blood (PBL) and lymphoblastoid cell lines obtained as described above were lysed and protein was measured. Samples of 20 ug protein of whole cell lysate were applied to each well of polyacrylamide gel. Electrophoresis was carried out under denaturing conditions (SDS-PAGE) in a 10% polyacrylamide gel. Protein was subsequently transferred to immobilon-P sheets and immunoblotted. A rabbit antiserum (Kally) which recognizes isoform II of ATPDase was used as primary antibody at a dilution of 1:10 000. The secondary antibody, a mouse monoclonal anti rabbit IgG conjugated to alkaline phosphatase (1:10 000) was detected by chemiluminescence using the Immun-Star™ substrate, according to the recommendations of the supplier (Bio-Rad/laboratories).

In vivo antibody production assay

Animal treatments and experimental design

[0065] Immunity mice were primed with antigen by an intraperitoneal injection of BSA as a T-dependent antigen. Briefly, BSA was resuspended in PBS (pH 7.2) and emulsified in the same volume of complete Freund's adjuvant (CFA). 30 ug BSA in 100 ml PBS was given to each mouse. Four groups of ten mice were immunized.

Group 1 : mice were injected i.p. with BSA alone with emulsified in (CFA) Group: Ag.

Group 2 : mice were injected i.p. with BSA plus BGO 136 (400mM) in 100 ml PBS, emulsified in (CFA) or erythrosin B (0.25 g/Kg)

Group 3 : mice were injected i.p. with BSA plus BGO 136 (800mM) in 100 ml PBS, emulsified in (CFA) or erythrosin B (0.5 g/Kg)

Group 4 : mice were treated with BSA in presence of the inhibitor emulsified in (CFA). These mice received various i.p. doses of BGO 136 400mM in 0.1 ml of normal PBS or erythrosin B 0.25 g/Kg, 2 doses/week are given as a maintenance dose. (group named DM = maintenance dose)

[0066] These initial immunizations (i.p.) were followed by two subsequent immunizations with emulsions of BSA alone or in presence of BGO136 400mM or 800mM, in incomplete Freund's adjuvant. Five days after each immunization, blood was collected from each animal, sera was analysed by an ELISA assay.

Detection of antibodies

[0067] Flat-bottomed microtiter plate were coated with BSA (5ug/ml) in $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$ 50mM (pH 9.6) at 37°C for 2 h. Plates were washed three times with (PBS buffer, pH 7.4, 0.5 ml/L Tween-20) and then blocked with 400 ul of milk per well overnight, at 4°C. Diluted sera specimens were added (100 ul/well) and incubated for 2h at 37°C. After thorough washing, wells were incubated with 100 ul of goat-anti-mouse IgG conjugated with peroxydase for 2 h at 37°C followed by several washes. TMB substrate solution (100 ul/well), (42mM TMB in DMSO, 0.1 M citric acid, Na_2HPO_4 0.2 M and H_2O_2 30%) was added. Color development was allowed to proceed for approximately 15 to 30 min, at room temperature. Reaction was terminated by adding 30ul of 4N H_2SO_4 . Optical density was read at 450 nm.

Cell growth and proliferation of Jurkat cell lines

[0068] T cell Jurkat is an acute T cell leukemia obtained from the peripheral blood of human. It was purchased from ATCC and was grown and maintained in RPMI™ 1640 medium containing 10% FBS, 2mM L-glutamine

adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES and 1.0 mM sodium pyruvate. To evaluate the effect of the different inhibitors of NTPDases on the growth of Jurkat cells, BGO 136, erythrosin, 8 Bus ATP, AMP and β - γ -Me ATP were added in the RPMI™ 1640 medium in presence of cells at day = 0. Cells counts and viability of cells were determined on day = 5.

Statistical analysis

[0069] Mice were used as test animals. Studies were also performed on the cultures of peripheral blood lymphocytes of donors and on experimental tumor cell lines. Test results were statistically processed. A one way variance analysis was followed; the Statpack® software analysis program was utilized.

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$

EXAMPLE 1

Presence of NTPDase in various normal and neoplastic lymphoid cells and inhibitory activity of NaN₃

[0070] The presence and activity of NTPDase in PBL cells and in various tumor cells were compared using ATP and ADP as substrates.

[0071] As may be seen in Figure 1, the NTPase signal is more pronounced in neoplastic cells as compared to that in normal lymphocytes (peripheral blood lymphocytes (PBL)).

[0072] The levels of the ecto-ATPase and ecto-ADPase functions of NTPDase are presented in Table 1 below. In agreement with the Western-blot

shown in Figure 1, the levels of NTPDase activity in normal PBL cells is relatively low as compared to that in tested tumor cell lines. It is noteworthy that in most cases, 10 mM Na azide (NaN₃) (a NTPDase inhibitor) causes more than 50% inhibition of enzyme activity.

Table 1 NTPDase activity of various intact human normal and tumoral lymphoid cells

		(nmoles Pi/min/10 ⁶ cells)			
		ATP	ATP + NaN ₃	ADP	ADP + NaN ₃
Cancer	Ramos	20 ± 0.3 ***	10 ± 0.0	14.5 ± 2.4 ***	8 ± 0
	Raji	39 ± 6.0 ***	14 ± 0.0	21 ± 0.8 ***	14 ± 0
	JurKat	25 ± 1.5 ***	7 ± 1.0	9.5 ± 0.8 ***	3.5 ± 0.8
	Mono Mac-1	23 ± 1.0 ***	7 ± 1.2	7.7 ± 0.2 ***	3.8 ± 1.8
	U937	29 ± 1.0 ***	8 ± 0.0	11 ± 0.5 ***	3.3 ± 0.8
Normal	PBL	1.0 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.2 ± 0.1

n = number of experiments (each in triplicate)

*** indicates that the difference of NTPDase activity between tumor cell lines group and the control PBL group is significant.

EXAMPLE 2

Effect of BGO 136 on NTPDase activity of PBL

[0073] As shown in figure 2, with PBL, BGO 136 caused a dose-dependent inhibition of ATPase and ADPase activities. A significant inhibition (40%) was obtained with 5 mM BGO 136 whereas with 10 mM, it increased up to 50% or more. The results are expressed as percent of controls considering untreated control samples as 100.

[0074] The influence of BGO 136 on human lymphocyte proliferation stimulated by ConA™ and LPS™ is shown in figure 3. ConA™ is a mitogen specific to lymphocytes T while LPS™ is a mitogen specific to lymphocytes B. BGO 136 did not affect the proliferation of non-activated lymphocytes as measured by 3H-Thymidine incorporation in DNA. In contrast, proliferation induced by ConA™ was inhibited in a dose-dependent manner, reaching about 10% of the positive control values with 10 mM BGO 136. The BGO 136 inhibition of LPS™-induced proliferation was much less pronounced. One has to take into account that cell-induced proliferation as expected is relatively low.

[0075] In PBL proliferation assays presented in the Examples below, ConA™ only was used to specifically stimulate T lymphocyte proliferation.

[0076] A positive correlation (.89 for ATPase and .96 for ADPase) between cell proliferation presented in Figure 3 and NTPDase activity presented in Figure 2 was established in the presence of the three concentrations of BGO 136 is illustrated in figure 4.

EXAMPLE 3

Effect of BGO 136 on humoral response

[0077] As shown in Figure 5, the levels of albumin antibodies were reduced as much as 50% by a single dose BGO 136 (800mM). Repeated injections of BGO 136 appeared even more efficient (group DM in Figure 5).

[0078] The experiments were repeated in several series of mice and produced essentially the same pattern of inhibition. The results from one representative experiment are presented in Figure 5.

[0079] No toxic or allergic reactions were observed during the BGO 136

administration (two months).

EXAMPLE 4

Effect of erythrosin B on PBL NTPDase activity and PBL proliferation

[0080] The effect of erythrosin B, a NTPDase inhibitor not structurally related to BGO 136, on lymphocyte T activity was also observed to determine its ability to influence immune response of T-cells. Results presented in Figures 6-7-8 show a dose-dependent inhibition of lymphocyte proliferation (Figure 7) and NTPDase activity (Figure 6). A high correlation was established between these two parameters (Figure 8).

EXAMPLE 5

Effect of erythrosin B on humoral response

[0081] As shown in figure 9, a significant decrease in the levels of antibodies to albumin following administration of erythrosin B can be observed especially with multiple injections to sustain the concentration of the inhibitor in the animal (group DM in Figure 9).

EXAMPLE 6

Effect of 8 BUS-ATP on PBL NTPDase activity and PBL proliferation

[0082] A pronounced dose-dependent inhibition of the NTPDase activity by 8 BUS-AMP was observed (Figure 10). 8 BUS-ATP reduced cell proliferation induced by ConA™ (Figure 11). A very good correlation could be established between these two parameters (Figures 12).

EXAMPLE 7

Effect of 8 BUS-AMP on PBL NTPDase activity and PBL proliferation

[0083] A pronounced dose-dependent inhibition of the NTPDase activity by 8 BUS-AMP was observed (Figure 13). 8 BUS-ATP reduced cell proliferation induced by ConA™ (Figure 14). A high correlation was established between these two parameters (Figures 15).

EXAMPLE 8**Effect of β - γ -Me ATP on PBL NTPDase activity and PBL proliferation**

[0084] As shown in figure 16, NTPDase was inhibited in a dose-dependent manner. β - γ -Me ATP reduced cell proliferation induced by ConA™ (Figure 17). Again, a high correlation was established between these two parameters (Figure 18).

EXAMPLE 9**Effect of BGO 136, erythrosin, 8 BUS ATP, AMP and β - γ -Me ATP on Jurkat cells' proliferation**

[0085] Figure 19 shows the influence of the different inhibitors on neoplastic T lymphocytes, namely Jurkat cells which are derived from human leukemia cells. BGO 136, erythrosin, 8 BUS ATP and AMP exerted a marked reduction of cell proliferation. A less pronounced but significant effect was observed with β - γ -Me ATP.

EXAMPLE 10**Comparison of effect of NTPDase inhibitors with compound of the prior art on graft vs. host reaction**

[0086] Table 2 below shows the efficiency of NTPDase inhibitors in preventing mice spleen growth following cell transplant according to the Bundick model. Mice were injected their parents' spleen cells. 21 days later, their spleen was weighed. Activation of Th2 (T helper cells) cells. The treatment groups are cyclosporin, BGO, and AMP. Bold figures in Table 2 correspond to results originally obtained by Bundick. The "No treatment group" is constituted of normal mice. The "positive" group is constituted of mice having undergone spleen tissue transplantation and having been injected with the carrier only (PBS™) without inhibitor. The other groups are constituted of mice having undergone spleen tissue transplantation and having been injected with the BGO 136, AMP or the cyclosporine, respectively. The spleen of all mice was then removed and weighed. The weigh of treated mice was then compared with that of the "No treatment" mice. Transplanted spleens of mice treated with

cyclosporine and BGO 136 are significantly smaller than those of the positive control mice (ANOVA < 5).

[0087] As may be seen in this table, BGO 136 was more efficient than cyclosporine in preventing spleen growth following transplantation.

TABLE 2 INFLUENCE OF VARIOUS INHIBITORS ON SPLEEN WEIGH AFTER TRANSPLANTATION

Groups	Mice	splenomegaly mg/ g of b. weight	suppression %
No treatment	6	2.1+0.23 <u>2.7+0.35</u>	100
Positive	8	7.1+1.11 <u>7.7+1.65</u>	0
BGO	8	4.3+ 0.29	56
AMP	8	6.7+ 0.82	8
Cyclosporine	6	5.1+ 0.09	40

CONCLUSION

[0088] The present invention showed a close correlation between inhibition of NTPDase activity and inhibition of immune responses of normal immune cells and neoplastic immune cells. All tested NTPDase inhibitors (i.e. BGO 136, erythrosin B, 8 Bus ATP, 8 Bus AMP and β - γ -Me ATP) decrease the NTPDase activity, and lymphocytic activity including proliferation and humoral response in normal T lymphocyte.

[0089] All the NTPDase inhibitors tested herein were shown to inhibit the growth of leukemic T lymphocyte cells (Jurkat cells) in culture.

[0090] As was seen in Table 1, the NTPDase activity in neoplastic

lymphoid cells is 10 times higher than that in normal lymphocytes (exemplified herein by PBL). NTPDase is therefore inducible in tumoral lymphoid cells. It is reasonably predictable therefore that since NTPDase inhibitors were able to inhibit humoral response in PBL cells, it will *a fortiori* inhibit this response in tumoral lymphoid cells.

[0091] Mice were shown to be a reliable and predictive model for human in the use of immunosuppressive agents in graft transplant (i.e. cyclosporine). The graft transplant rejection mechanism therefore appears to be similar in mice and human. Furthermore, with regards particularly to NTPDase, as was shown in Benrezzak *et al.* 2000, human, mice (and pigs) have very similar biochemical profiles with regards to NTPDase activity and localization. Results presented herein obtained with mice may reasonably predict that similar results will be obtained in human for graft transplant and inhibition of immune cell activity involving NTPDase activity.

[0092] Although the present invention has been described hereinabove by way of preferred embodiments thereof, it can be modified, without departing from the spirit and nature of the subject invention as defined in the appended claims.

REFERENCES

- [1] Abbrachio, M.P. and Burnstock, G. (1998) *Jpn. J. Pharmacol.* 78, 113-145.
- [2] Williams, M. and Jarvis, M.J. (2000) *Biochem. Pharmacol.* 59, 1173-1185.
- [3] Drury, A.N. and Szent-Györgyi, A. (1929) *J. Physiol. Lond.* 68, 23-237.
- [4] Bennet, D.W. and Drury, A.N. (1931) *J. Physiol. Lond.* 72, 288-320.
- [5] Holton, F.A. and Holton, P. (1954) *J. Physiol. Lond.* 126, 124-140.
- [6] Holton, P. (1959) *J. Physiol. Lond.* 145, 494-504.
- [7] Burnstock, G. (1969) *Pharmacol. Rev.* 21, 247-324.
- [8] Su, C.; Bevan, J.A. and Burnstock, G. (1971) *Science* 173, 336-338.
- [9] Langer, S.Z. and Pinto, E.B. (1976) *J. Pharmacol. Exp. Ther.* 196, 697-713.
- [10] Burnstock, G. (1976) *J. Theor. Biol.* 62, 491-503.
- [11] Burnstock, G. (1971) *Nature* 229, 282-283.
- [12] Burnstock, G. (1978) In: *Cell membrane receptors for drugs and hormones: A multidisciplinary approach*, (Straub, R.W. and Bolis, L.; Eds.), Raven Press, New York, pp. 108-118.
- [13] Coade, S.B. and Pearson, J.D. (1989) *Circ. Res.* 65, 531-537.
- [14] Beaudoin, a.r.; grondin, G.; enjyoji, K.; robson, S.C.; sévigny, J.; fischer, B. and gendron, F.P. (2000) in: *Ecto-ATPases and related ectonucleotidases*. (Vanduffel, L. and Lemmens, R.; Eds), Shaker Publishing B.V.; The Netherlands, pp. 125-135.
- [15] Burnstock, G. (1972) *Pharmacol. Rev.* 24, 510-569.
- [16] Lloyd, h.g.e. and stone, T.W. (1983) *Proc. Physiol. Soc.* 57p.
- [17] White, T.D. (1988) *Pharmacol. Ther.* 38, 129-168.
- [18] Williams, M. (1990) *Ann. N. Y. Acad. Sci.* 603, 93-107.
- [19] Sneddon, P. and Burnstock, G. (1984) *Eur. J. Pharmacol.* 100, 85-90.
- [20] Sneddon, P. and Westfall, D.P. (1984) *J. Physiol. Lond.* 347, 561-580.
- [21] Burnstock, G. (1986) *Prog. Brain Res.* 68, 193-203.
- [22] Kirkpatrick, K. and burnstock, G. (1987) *Eur. J. Pharmacol.* 138, 207-214.
- [23] Vizi, E.S. and Burnstock, G. (1988) *Eur. J. Pharmacol.* 158, 69-77.
- [24] Westfall, D. P.; Sedaa, K.O.; Shinozuka, K.; Bjun, R.A. and Buxton, I.L. (1990) *Ann. N. Y. Acad. Sci.* 603, 300-310.
- [25] Von Kugelgen, I. and Starke, K. (1994) *Naunyn-Schmiedeberg's Arch. Pharmacol.* 350, 123-129.
- [26] Kennedy, C.; McLaren, G.J.; Westfall, T.D. and Sneddon, P. (1996) *Ciba Foundation Symposium* 198, 223-235.
- [27] Brock, J.A.; Bridgewater, M. and Cunnane, T.C. (1997) *Br. J. Pharmacol.* 120, 769-776.

- [28] Richardson, P.J. and Brown, S.J. (1987) *J. Neurochem.* 48, 622-630.
- [29] Ding, Y.; Cesare, P.; Drew, L.; Nikitaki, D. and Wood, J.N. (2000) *J. Auton. Nerv. Syst.* 81, 289-294.
- [30] Hamilton, S.G. and McMahon, S.B. (2000) *J. Auton. Nerv. Syst.* 81, 187-194.
- [31] Hamilton, S.G.; Wade, A. and McMahon, S.B. (1999) *Br. J. Pharmacol.* 126, 326-332.
- [32] Unsworth, c.d. and johnson, R.G. (1990) *Ann. N. Y. Acad. Sci.* 603, 353-363.
- [33] Uvnas, B. (1974) *Life Sci.* 14, 2355-2366.
- [34] Roman, R.M.; wang, Y.; lidofsky, S.D.; feranchak, A.P.; lomri, N.; scharschmidt, B.F. and fitz, J.G. (1997) *J. Biol. Chem.* 272, 21970-21976.
- [35] Sprague, r.s.; ellsworth, M.L.; stephenson, A.H.; kleinhenz, M.E. and lonigro, A.J. (1998) *Am. J. Physiol.* 44, H1726-H1732.
- [36] Sugita, m.; yue, Y. and foskett, J.F. (1998) *EMBO J.* 17, 898-908.
- [37] Beaudoin, a.r.; grondin, G. and gendron, F.P. (1999) *Prog. Brain Res.* 120, 387-395.
- [38] Taylor, a.l.; kudlow, B.A.; maus, K.L.; gruenert, D.C.; guggino, W.B. and schwiebert, E.M. (1998) *Am. J. Pharmacol.* 275, C1391-C1406.
- [39] Wilson, p.d.; hovater, J.S.; casey, C.C.; fortentberry, J.A. and schwiebert, E.M. (1999) *J. Am. Soc. Nephro.* 10, 219-229.
- [40] Schlosser, S.F.; Burgstahler, A.D. and Nathanson, M.H. (1996) *Proc. Natl. Acad. Sci. USA* 93, 9948-9953.
- [41] Lidofsky, S. (1997) *Hepatology* 25, 778-779.
- [42] Queiroz, g.; gebicke-haerter, P.J.; schobert, A.; starke, K. and Von kugelgen, L. (1997) *Neurosci.* 78, 1203-1208.
- [43] Guthrie, p.b.; knappenberger, J.; segal, M.; bennett, M.V.; charles, A.C. and kater, S.B. (1999) *J. Neurosci.* 19, 520-528.
- [44] Rojas, e.; pollard, H.B. and heldman, E. (1985) *FEBS Letters* 185, 323-327.
- [45] Xu, y.p.; duarte, E.P. and forsberg, E.J. (1991) *J. Neurochem.* 56, 1889-1896.
- [46] Weil-malherbe, h. and bone, A.D. (1958) *Biochem. J.* 70, 14-22.
- [47] Marquardt, d.l.; gruber, H.E. and wasserman, S.I. (1994) *Proc. Natl. Acad. Sci. USA* 81, 6192-6196.
- [48] Rapaport, e. and fontaine, J. (1989) *Biochem. Pharmacol.* 38, 4261-4266.
- [49] Sperlagh, b.; hasko, G.; nemeth, Z. and vizi, E.S. (1998) *Neurochem. Int.* 33, 209-215.
- [50] Fredholm, B. B.; Abbracchio, M.P.; G. Burnstock, G.; Daly, J.W.; Harden, T.K.; Jacobson, K.A.; Leff, P. and Williams, M. (1994) *Pharmacol. Rev.* 46, 143-156.
- [51] Tucker, A.L. and Linden, J. (1993) *Cardiovasc. Res.* 27, 62-67.
- [52] Dubyak, G. R. and El-Moatassim, C. (1993) *Am. J. Physiol* 265,

C577-C606.

- [53] Stoop, R.; Thomas, S.; Rashedren, F.; Kawashima, E.; Buell, G.; Surprenant, A. and North, R. (1999) *Mol. Pharmacol.* 56, 973-981.
- [54] Khakh, B.S.; Burnstock, G.; Kennedy, C.; King, B.F.; North, R.A.; Séguéla, P.; Voigt, M. and Humphrey, P.P.A. (2001) *Pharmacol. Rev.* 53(1), 107-118.
- [55] Weisman, G.A.; Turner, J.T.; Clarke, L.L.; Gonzalez, F.A.; Otero, M.; Garrad, R.C. and Erb, L. (1997) in: *Ecto-ATPases*, (Plesner, L.; Kirley, T.L. and Knowles, A.F.; Eds.), Plenum Press. New York, USA, pp. 231-237.
- [56] Evans, R.J.; Lewis, C.; Virgilio, C.; Lundstrom, K.; Buell, G.; Surprenant, A. and North, R.A. (1996) *J. Physiol. Lond.* 497, 413-422.
- [57] Vulchanova, L.; arvidsson, U.; riedl, M.; wang, J.; buell, G.; surprenant, A.; north, R.A. and elde, R. (1996) *Proc. Natl. Acad. Sci. USA* 93, 8063-8067.
- [58] Boarder, m.r. and hourani, s.m.o. (1998) *Trends Pharmacol. Sci.* 19, 99-107.
- [59] Nicke, A.; Baumert, H.G.; Rettinger, J.; Eichele, A.; Lambrecht, G.; Mutschler, E. and Schmalzing, G. (1998) *EMBO J.* 17, 3016-3028.
- [60] Fedan, J. S.; Dagirmanjian, J.P.; Attfield, M.A. and Chideskel, E.W. (1990) *J. Pharmacol. Exp. Ther.* 255, 46-51.
- [61] Nakazawa, K.; Fujimori, K.; Takanakna, A. and Inoue, K. (1990) *J. Physiol. Lond.* 428, 257-272.
- [62] Sela, D.; Ram, E. and Atlas, D. (1991) *J. Biol. Chem.* 266, 17990-17994.
- [63] Berridge, M. J. and Irvine, R.F. (1989) *Nature* 341, 197-205.
- [64] Gordon, J.L. (1986) *Biochem. J.* 233, 309-319.
- [65] Surprenant, A.; Rassendren, F.; Kawashima, E.; North, R.A. and Buell, G. (1996) *Science* 272, 735-738.
- [66] Ferrari, D.; Chiozzi, P.; Falzoni, S.; Dal Susino M.; Melchiorri, L.; Baricordi, O.R. and Di Virgilio, F. (1997) *J. Immunol.* 15, 1451-1458.
- [67] Solle, M.; Labasi, J.; Perregaux, D.G.; Stam, E.; Petrushova, N.; Koller, B.H.; Griffiths, R.J. and Gabel, C.A. (2001) *J. Biol. Chem.* 276(1), 125-132.
- [68] Sneddon, P.; Westfall, T.D.; Todorov, L.D.; Mihaylova-Todorova, S.; Westfall, D.P. and Kennedy, C. (1999) *Prog. Brain Res.* 120, 11-20.
- [69] Kennedy, C. and Leff, p. (1995) *Trends Pharmacol. Sci.* 16, 168-174.
- [70] North, R.A. (1996) *Sem. Neurosci.* 8, 187-194.
- [71] Torres, G.; Egan, T. and Voigt, M. (1999) *J. Biol. Chem.* 274, 6653-6659.
- [72] Lewis, C.; Neidhart, S.; Holy, C.; North, R.A.; Buell, G. and Surprenant, A. (1995) *Nature (Lond.)* 377, 432-435.
- [73] Cook, S.P.; Vulchanova, L.; Hargreaves, K.M.; Elde, R. and McCleskey, E.W. (1997) *Nature (Lond.)* 387, 505-508.
- [74] Radford, K.M.; Virginio, C.; Surprenant, A.; North, R.A. and

- Kawashima, E. (1997) *J. Neurosci.* 17, 6529-6533.
- [75] Vulchanova, L.; Riedl, M.S.; Shuster, S.J.; Buell, G.; Surprenant, A.; North, A.R. and Elde, R. (1997) *Neuropharmacology* 36, 1229-1242.
- [76] Haines, W.R.; Torres, G.E.; Voigt M.M. and Egan, T.M. (1999) *Mol. Pharmacol.* 56, 720-727.
- [77] Le, K.T.; Boue-Grabot, E.; Archambault V. and Séguéla, P. (1999) *J. Biol. Chem.* 274, 15415-15419.
- [78] Torres, G.E.; Egan, T.M. and Voigt, M.M. (1998) *Biochemistry* 37, 4845-4851.
- [79] Le, K.T.; Babinski, K. and Séguéla, P. (1998) *J. Neurosci.* 18, 7152-7159.
- [80] Hourani, S.M.O. and Hall, D.A. (1994) *Trends Pharmacol. Sci.* 15, 103-108.
- [81] Communi, D.; Govaerts, C.; Parmentier, M. and Boeynaems, J.M. (1997) *J. Biol. Chem.* 272, 31969-31973.
- [82] Leon, C.; Hechler, B.; Vial, C.; Leray, C.; Cazenave, J.-P. and Gachet, T.C. (1997) *FEBS Lett.* 403, 26-30.
- [83] Nicholas, R.A.; Watt, W.C.; Lazarowski, E.R.; Li, Q. and Harden, T.K. (1996) *Mol. Pharmacol.* 50, 224-229.
- [84] Boarder, M.R.; Weisman, G.A.; Turner, J.T. and Wilkinson, G.F. (1995) *Trends Pharmacol. Sci.* 16, 133-138.
- [85] Harden, T.K.; Boyer, J.L. and Nicholas, R.A. (1995) *Ann. Rev. Pharmacol. Toxicol.* 35, 541-579.
- [86] Yang, C.M.; Tsai, Y.-J.; Pan, S.-L.; Tsai, C.-T.; Wu, W.-b.; Chiu, C.-T.; Luo, S.-F. and Ou, J.T. (1997) *Naunyn-Schmiedeberg's Arch. Pharmacol.* 356, 1-7.
- [87] Erb, L.; Lustig, K.D.; Sullivan, D.M.; Turner, J.T. and Weisman, G.A. (1994) *Proc. Natl. Acad. Sci. USA* 90, 10449-10453.
- [88] Boyer, J.L.; Delaney, S.M.; Villanueva, D. and Harden, T.K. (2000). *Mol. Pharmacol.* 57, 805-810
- [89] Hollopeter, G.; Jantzen, H.M.; Vincent, D.; Li, G.; England, L.; Ramakrishnan, V.; Yang, R.B.; Nurden, P.; Nurden, A.; Julius, D. and Conley, P.B. (2000) *Nature* 409, 202-207.
- [90] Tu, M.-T.; Luo, S.-F.; Wang, C.-C.; Chien, C.-S.; Chiu, C.-T.; Lin, C.-C. and Yang, C.M. (2000) *Br. J. Pharmacol.* 129, 1481-1489.
- [91] Erb, L.; Liu, J.; Ockerhausen, J.; Kong, Q.; Garrad, R.C.; Griffin, K.; Neal, C.; Krugh, B.; Santiago-Pérez, L.I.; Gonzalez, F.A.; Gresham, H.D.; Turner, J.T. and Weisman, G.A. (2001) *J. Cell. Biol.* 153, 491-501.
- [92] Short, S.M.; Boyer, J.L. and Juliano, R.L. (2000) *J. Biol. Chem.* 275, 12970-12977.
- [93] Zimmermann, H. (2000) *Naunyn-Schmiedeberg's Arch. Pharmacol.* 362, 299-309.
- [94] Enjyoji, K.; Sevigny, J.; Lin, Y.; Fenette, P.S.; Christie, P.D.; Schulte am Esch II, J.; Imai, M.; Edelberg, J.M.; Rayburn, H.; Lech, M.; Beller, D.L.;

- Csizmadia, E.; Wagner, D.D.; Robson, S.C. and Rosenberg, R.D. (1999) *Nature Med.* 5, 101-1017.
- [95] LeBel, D.; Poirier, G.G.; Phaneuf, S.; St-Jean, P.; Laliberté, J.-F. and Beaudoin, A.R. (1980) *J. Biol. Chem.* 255, 1227-1233.
- [96] Zimmermann, H. (1992) *Biochem. J.* 285, 345-365.
- [97] Ziganshin, A.U.; Hoyle, C.H.V and Burnstock, G. (1994) *Drug Dev. Res.* 32, 134-146.
- [98] Plesner, L. (1995) *Int. Rev. Cytol.* 158, 141-214.
- [99] Beaudoin, A. R.; Sévigny, J. and Picher, M. (1996) In: *Biomembranes Vol.5.* (Lee, A.G.; Ed.). JAI Press Inc. Greenwich, pp. 367-399.
- [100] Vlajkovic, S.M.; Thorne, P.R.; Hously, G.D.; Munoz, D.J.B. and Kendrick, I.S. (1998) *Neuroreport* 9, 1559-1565.
- [101] Culic, O.; Sabolic, I. and Zanic-Grubisic, T. (1990) *Biochim. Biophys. Acta* 1030, 143-151.
- [102] Meyerhof, O. (1945) *J. Biol. Chem.* 157, 105-119.
- [103] Kalckar, H.M. (1944). *J. Biol. Chem.* 153, 355-367.
- [104] Ribeiro, J.M.C. (1987) *Ann. Rev. Entomol.* 32, 463-478.
- [105] Ribeiro, J.M.C. (2000) *Med. Vet. Entom.* 14, 142-148.
- [106] Vasconcelos, E.G.; Nascimento, P.S.; Meirelles, M.N.L.; Verjovski-Almeida, S. and Ferreira, S.T. (1993) *Mol. Biochem. Parasitol.* 58, 205-214.
- [107] Anich, M.; Fanta, N.; Mancilla, M.; Kettlun, A.M, Valenzuela, M.A. and Traverso-Cori, A. (1990) *Phytochemistry* 29, 1411-1415.
- [108] Thomas, C.; Sun, Y.; Naus, K.; Lloyd, A. and Roux, S. (1999) *Plant Physiol.* 119, 543-551.
- [109] Bhargava, T.; Datta, S.; Ramachandran, V.; Ramakrishnan, R.; Roy, R.K.; Sankaran, K. and Subrahmanyam, Y.V.B.K. (1995) *Curr. Sci. India* 68, 293-300.
- [110] Curdova, E.; Jechova, V. and Hostalek, Z. (1982) *Folia Microbiol.* 27, 159-166.
- [111] Trilisenko, L.V.; Novotna, J.; Erban, V.; Behal, V. and Hostalek, Z. (1987) *Folia Microbiol.* 32, 402-410.
- [112] Vasconcelos, E.G.; Ferraira, S.T.; De Carvalho, T.M.U.; De Souza, W.; Kettlun, A.M.; Mancilla, M.; Valenzuela, M.A. and Verjovski-Almeida, S. (1996) *J. Biol. Chem.* 271, 22139-22145.
- [113] Berlutti, F.; Casalino, M.; Zagaglia, C.; Fradiana, P.A.; Visca, P. and Nicoletti, M. (1998) *Inf. Immun.* 66, 4957-4964.
- [114] Yagi, K.; Arai, Y.; Kato, N.; Hirota, K. and Miura, Y. (1989) *Eur. J. Biochem.* 180, 509-513.
- [115] Côté, Y.-P.; Picher, M.; St-Jean, P.; Béliveau, R.; Potier, M. and Beaudoin, A.R. 1991. *Biochim. Biophys. Acta* 1078, 187-191.
- [116] Beaudoin, A. R.; Sévigny, J.; Grondin, G.; Daoud, S. and Levesque, F.P. (1997) *Am. J. Physiol.* 273: H673-H681.
- [117] Sévigny, J.; Levesque, F.P.; Grondin, G. and Beaudoin, A.R. (1997). *Biochim. Biophys. Acta* 1334, 73-88

- [118] Picher, M.; Côté, Y.P.; Béliveau, R.; Potier, M. and Beaudoin, A.R. (1993) *J. Biol. Chem.* 268, 4699-4703.
- [119] Sévigny, J.; Picher, M.; Grondin, G. and Beaudoin, A.R. (1997) *Am. J. Physiol.* 272, L939-L950.
- [120] Sévigny, J.; Grondin, G.; Gendron, F.P.; Roy, J. and Beaudoin, A.R. (1998) *Am. J. Physiol.* 275, G473-G482.
- [121] Leclerc, M.-C.; Grondin, G.; Gendron, F.P.; Sévigny, J. and Beaudoin, A.R. (2000) *Arch. Biochem. Biophys.* 377, 373-378.
- [122] Sévigny, J.; Robson, S.C.; Waelkens, E.; Csizmadia, E.; Smith, R.N. and Lemmens, R. (2000) *J. Biol. Chem.* 275, 5640-5647.
- [123] Zimmermann, H. and Braun, N. (1996) *J. Auton. Pharmacol.* 16, 397-400.
- [124] Wang, T.F. and Guidotti, G. (1998) *Brain Res.* 790, 318-322.
- [125] Maliszewski, C.R.; Delespesse, G.J.T.; Schoenborn, M.A.; Armitage, R.J.; Fanslow, W.C.; Nakajima, T.; Baker, E.; Sutherland, G.R.; Poindexter, K.; Birks, C.; Alpert, A.; Friend, D.; Gimpel, S.D. and Gayle III, R.B. (1994) *J. Immunol.* 153, 3574-3583.
- [126] Benrezzak, O.; Grondin, G.; Sévigny, J.; Gendron, F.P.; Rousseau, E.; D'Orléans-Juste, P. and Beaudoin, A.R. (1999) *Arch. Biochem. Biophys.* 370, 314-322.
- [127] Valenzuela, M. A.; Lopez, J.; Depix, M.; Mancilla, M.; Kettlun, A.M.; Catalan, L.; Chiong, M.; Garrido, J. and Traverso-Cori, A. (1989) *Comp. Biochem. Physiol.* 93B, 509-513.
- [128] Papamarcaki, T. and Tsolas, O. (1990) *Mol. Cell. Biochem.* 97, 1-8.
- [129] Magocsi, M. and Penniston, J.T. (1991) *Biochim. Biophys. Acta* 1070, 163-172.
- [130] Pieber, M.; Valenzuela, M.A.; Kettlun, A.M.; Mancilla, M.; Aranda, E.; Collados, L. and Traverso-Cori, A. (1991) *Comp. Biochem. Biophys.* 100B, 281-285.
- [131] Lemmens, R.; Kupers, L.; Sévigny, J.; Beaudoin, A.R.; Grondin, G.; Kittel, A.; Waelkens, E. and Vanduffel, L. (2000) *Am. J. Physiol.* 278, F978-F988.
- [132] Handa, M. and Guidotti, G. (1996) *Biochem. Biophys. Res. Comm.* 218, 916-923.
- [133] Schulte, J.A.E., Sévigny, J.; Kaczmarek, E.; Siegel, J.B.; Imai, M.; Koziak, K.; Beaudoin, A.R. and Robson, S.C. (1999) *Biochemistry* 38, 2248-2258.
- [134] Smith, T.M.; Carl, S.A.L. and Kirley, T.L. (1999) *Biochemistry* 38, 5849-5857.
- [135] Drosopoulos, J.H.F.; Broekman, M.J.; Islam, N.; Maliszewski, C.R.; Gayle, R.B. and Marcus, A.J. (2000) *Biochemistry* 39, 6936-6943.
- [136] Grinthal, A. and Guidotti, G. (2000) *Biochemistry* 39, 9-16.
- [137] Asai, T.; Miura, S.; Sibley, L.D.; Okabayashi, H. and Takeuchi, T. (1995) *J. Biol. Chem.* 270, 11391-11397.

- [138] Kegel, B.; Braun, N.; Heine, P.; Maliszewski, C.R. and Zimmermann, H. (1997) *Neuropharmacol.* 36, 1189-1200.
- [139] Stout, J.G. and Kirley, T.L. (1996) *Biochemistry* 35, 8289-8298.
- [140] Wang, T.F.; Ou, Y. and Guidotti, G. (1998) *J. Biol. Chem.* 273, 24814-24821.
- [141] Wang, T.F.; Rosenberg, P.A. and Guidotti, G. (1997) *Brain Res. Mol. Brain Res.* 47, 395-302.
- [142] Sévigny, J.; Dumas, F. and Beaudoin, A.R. (1997) In: *Ecto-ATPase. Recent progress on structure and function.* (Plesner, L.; Kirley, T.L. and Knowles, A.F.; Eds.) Plenum Press. New York, 294 P.
- [143] Chadwick, B.P. and Frischauf, A.-M. (1998) *Genomics* 50, 357-367.
- [144] Schoenborn, M.A.; Jenkins, N.A.; Copeland, N.G.; Gilbert, P.J.; Gayle III, R.B. and Maliszewski C.R. (1998) *Cytogenet. Cell Genet.* 81, 287-289.
- [145] Zimmermann, H.; Beaudoin, A.R.; Bollen, M.; Goding, J.W.; Guidotti, G.; Kirley, T.L.; Robson, S.C. and Sano, K. (2000). In: *Ecto-ATPases and related ectonucleotidases.* (Vanduffel, L. and Lemmens, R.; Eds) Shaker Publishing B.V.; The Netherlands. Pp 1-8.
- [146] Côté, Y.P.; Filep, J.G.; Battistini, B.; Gauvreau, J.; Sirois, P. and Beaudoin, A.R. (1992) *Biochim. Biophys. Acta* 1139, 133-42.
- [147] Kirley, T.L. (1997) *J. Biol. Chem.* 272, 1076-1081.
- [148] Marcus, A.J.; Broekman, M.J.; Drosopoulos, J.H.F.; Islam, N.; Alyonycheva, T.N.; Safier, J.B.; Hajjar, K.A.; Posnett, D.N.; Schoenborn, M.A.; Schooley, K.A.; Gayle III, R.B. and Maliszewski, C.R. (1997) *J. Clin. Invest.* 99, 1351-1360.
- [149] Koziak, K.; Sévigny, J.; Robson, S.C.; Siegel, J.B. and Kaczmarek, E. (1999) *Thromb. Haemo.* 82, 1538-1544.
- [150] Kansas, G.S.; Wood, G.S. and Tedder, T.F. (1991) *J. Immunol.* 146, 2235-2244.
- [151] Frassetto, S.S.; Dias, R.D. and Sarkis, J.J.F. (1993) *Mol. Cell. Biochem.* 129, 47-55.
- [152] Marcus, A.J. (1996) In: *Disorders of Hemostasis, chap.4* (O.D. Ratnoff and C.D. Forbes, ed.), pp. 79-137, Philadelphia, U.S.A.
- [153] Hirota, K.; Saski, N.; Yagi, K. and Miura, Y. (1987) *Thromb. Res.* 45, 201-209.
- [154] Kaczmarek, E.; Koziak, K.; Sévigny, J.; Siegel, J.B.; Anrather, J.; Beaudoin, A.R.; Bach, F.H. and Robson, S.C. (1996) *J. Biol. Chem.* 271, 33116-33122.
- [155] Koyamada, N.; Miyatake, T.; Candinas, D.; Hechenleither, P.; Siegel, J.; Hancock, W.W.; Bach, F.H. and Robson, S.C. (1996) *Transplantation* 62, 1739-1743.
- [156] Imai, M.; Takigami, K.; Guckelberger, O.; Enjoji, K.; Neal Smith, R.; Lin, Y.; Csizmadia, E.; Sévigny, J.; Rosenberg, R.D.; Bach, F.H. and Robson, S.C. (1999) *Mol. Med.* 5, 743-752.
- [157] Braun, N.; Zhu, Y.; Kriegstein, J.; Culmsee, C. and Zimmermann, H.

1998. *J. Neurosci.* 18, 4891-4900.
- [158] Rongen, G.A.; Floras, J.S.; Lender, J.W.; Thien, T.; Smits, P. (1997) *Clin. Sci.* 92, 13-24.
- [159] Di Virgilio, F.; Bronte, V.; Collavo, D. and Zanovello, P. (1989) *J. Immunol.* 143, 1955-1960.
- [160] Filippini, A.; Taffs, R.E.; Agui, T. and Sitkovsky, M.V. (1990) *J. Biol. Chem.* 265, 334-340.
- [161] Antonyamy, M.A.; Moticka, E.J. and Ramkumar, V. (1995) *J. Immunol.* 155, 2813-2821.
- [162] Krishnaraj, R. (1992) *Cell Immunol.* 141, 306-322.
- [163] Krishnaraj, R. (1992b) *Cell Immunol.* 144, 11-21.
- [164] Bajpai, A. and Brahmi, Z. (1993) *Cell. Immunol.* 148, 130-143.
- [165] Dombrowski, K. E.; Cone, J.C.; Bjorndahl, J.M. and Phillips, C.A. (1995) *Cell. Immunol.* 160, 199-204.
- [166] Correale, P.; Giuliano, M.; Tagliaferri, P.; Guarrusi, R.; Caraglia, M.; Marinetti, M.R.; Iezzi, T.; Bianco, A.R. and Procopio, A. (1995) *Res. Comm. Mol. Pathol. Pharmacol.* 87, 67-69.
- [167] Imai, M.; Goepfert, C.; Kaczmarek E. and Robson, S.C. (2000) *Biochem. Biophys. Res. Comm.* 270, 272-278.
- [168] Clifford, E.E.; Martin, K.A.; Dalal, P.; Thomas, R. and Dubyak, G.R. (1997) *Am. J. Physiol.* 42, C973-C987.
- [169] Dzhandzhugazyan, K.N.; Kirkin, A.F.; Straten, P.T. and Zeuthen, J. (1998) *FEBS Lett.* 430, 227-230.
- [170] Gendron, F.P.; Halbfinger, E.; Fischer, B.; Duval, M.; D'Orleans-Juste, P. and Beaudoin, A. R. (2000a) *J. Med. Chem.* 43, 2239-2247.
- [171] Gendron, F.P.; Halbfinger, E.; Fischer, B.; and Beaudoin A.R. (2000) In: *Purine and pyrimidine metabolism in man X* (Eds. E. Zoref-Shani and O. Sperling). *Adv. Exp. Med. Biol.* 486, 119-123.
- [172] Picher, M.; Sévigny, J.; D'Orléans-Juste, P. and Beaudoin, A.R. (1996) *Biochem. Pharmacol.* 51, 1453-1460.
- [173] Fischer, B.; Chulkin, A.; Boyer, J.L.; Harden, K.T.; Gendron, F.P.; Beaudoin, A.R.; Chapal, J.; Hillaire-Buys, D. and Petit, P. (1999) *J. Med. Chem.* 42, 3636-3646.
- [174] Halbfinger, E.; Major, D.T.; Ritzman, M.; Ubl, J.J.; Reiser, G.; Boyer, J.L.; Harden, K.T. and Fischer, B. (1999) *J. Med. Chem.* 42, 5325-5337.
- [175] Fischer, B.; Kabha, E.; Gendron, F.P. and Beaudoin, A.R. (2000) *Nucleosides, Nucleotides & Nucleic Acid* 19, 1033-1054.
- [176] Bültmann, R.; Wittenburg, H.; Pause, B.; Kurz, G.; Nickel, P. and Starke, K. (1996) *Naunyn-Schmiedeberg's Arch. Pharmacol.* 354, 498-504.
- [177] Tuluc, F.; Bültmann, R.; Glänzel, M.; Wilhelm Frahm, A. and Starke, K. (1998) *Naunyn-Schmiedeberg's Arch. Pharmacol.* 357, 111-120.
- [178] Wittenburg, H.; Bültmann, R.; Pause, B.; Ganter, C.; Kurz, G. and Starke, K. (1996) *Naunyn-Schmiedeberg's Arch. Pharmacol.* 354, 491-497.
- [179] Bültmann, R.; Pause, B.; Wittenburg, H.; Kurz, G. and Starke, K.

- (1996) Naunyn-Schmiedeberg's Arch. Pharmacol. 354, 481-490.
- [180] Parr, C.E.; Sullivan, D.M.; Paradiso, A.M.; Larowski, E.R.; Burch, L.H.; Olsen, J.C.; Erb, L.; Weisman, G.A.; Boucher, R.C. and Turner, J.T. (1994) Proc. Nat. Acad. Sci. USA 91, 3275-3279.
- [181] Vljakovic, S.M.; Hously, G.D.; Greenwood, D. and Thorne PR. (1999) Mol. Brain Res. 73, 85-92.
- [182] Zimmermann, H. (1999) Nat. Med. 5, 987-988.
- [183] Bergfeld, g.r. and forrester, T. (1992) Cardiovas. Res. 26, 40-47.
- [184] Knoefler, r.; Weissbach, G. and Kuhlisch, E. (1997) Am. J. Hematol. 56, 259-265.
- [185] Detwiler, t.c. and feinman, R.D. (1973) Biochemistry 12, 2462-2468.
- [186] Li, b.y. and li, W.H. (1998) Acta Pharmacol. Sinica 19, 383-386.
- [187] Bodin, p. and burnstock, G. (1995) Experientia 51, 256-259.
- [188] Bodin, p.; milner, P.; winter, R. and burnstock, G. (1992) Proc. R. Soc. Lond. 247, 131-135.
- [189] Schini, v.b.; hendrickson, H.; heublein, D.M.; burnett, J.C. and vanhoutte, P.M. (1989) Eur. J. Pharmacol. 165, 333-334.
- [190] Bodin, p. and burnstock, G. (1996) J. Cardiovasc. Pharmacol. 27, 872-875.
- [191] Sedaa, k.o.; bjur, R.A.; schinozuka, K. and westfall, D.P. (1990) J. Pharmacol. Exp. Ther. 252, 1060-1067.
- [192] Yang, s.; cheek, D.J.; westfall, D.P. and buxton, I.L. (1994) Circ. Res. 74, 401-407.
- [193] Vizi, e.s.; sperlagh, B. and burnstock, G. (1992) Neurosci. 50, 455-465.
- [194] Katsuragi, t.; tokunaga, T.; ogawa, S.; soejima, O.; sato, C. and furukawa, T. (1991) J. Pharmacol. Exp. Ther. 259, 513-518.
- [195] Katsuragi, t.; tamesue, S.; sato, C.; sato, Y. and furukawa, T. (1996) Naunyn-Schmiedeberg's Arch. Pharmacol. 354, 796-799.
- [196] Tamesue, s.; sato, C. and katsuragi, T. (1998) Naunyn-Schmiedeberg's Arc. Pharmacol. 357, 240-244.
- [197] Gendron F.P., Benrezzak, O., Krugh, B.W., Kong, Q., Weisman, G. A., and Beaudoin A.R. 2002. Purine signalling and potential new therapeutic approach: possible outcome of NTPDase inhibition. Current Drug Targets 3(3): 229-245.
- [198] Nahum, V., Zundorf, G., Reiser, G., Levesque, S.A., Beeaudoin, A.R., Fischer, B. 2002. 5'-O-(1-Boranotriphosphate)-Adenosine Derivatives as Novel P2Y₁-Receptor Agonists. (Accepted J. Med.Chem.)

WHAT IS CLAIMED IS:

1. A method of screening for a compound useful in the treatment of a disease or condition characterized by an immune cells disorder, wherein said cell expresses NTPDases, said method comprising the steps of

contacting a candidate compound with ecto-nucleoside triphosphate diphosphohydrolase (NTPDase),

wherein the candidate compound is selected if the activity of said NTPDase is reduced in the presence of the candidate compound as compared to that in the absence thereof.

2. A method as defined in claim 1, wherein said contacting of said candidate compound with said NTPDase is performed in an immune cell selected from the group consisting in normal T lymphocyte, normal B lymphocyte, normal NK cell, normal macrophage, normal monocyte, Jurkat cell, Raji cell, Ramos cell, MonoMac™ cell, K562 cell and U937 cell.

3. A method as defined in claim 2, wherein said immune cell is a T lymphocyte.

4. A method as defined in claim 2, wherein said immune cell is a B lymphocyte.

5. A method as defined in claim 2, wherein said immune cell is a Jurkat cell.

6. A method as defined in claim 2, wherein said immune cell is a Raji Cell.

7. A method as defined in claim 2, wherein said immune cell is a Ramos cell.

8. A method as defined in claim 2, wherein said immune cell is a MonoMac™ cell.

9. A method as defined in claim 2, wherein said immune cell is a K562 cell.

10. A method as defined in claim 2, wherein said immune cell is a U937 cell.

11. A method as defined in claim 2, wherein said immune cell is a NK cell.

12. A method for inhibiting an immune cell activity in a mammal, comprising targeting immune cells with an effective amount of a NTPDase inhibitor.

13. A method as recited in claim 12, wherein said cells are normal lymphocytes.

14. The method as recited in claim 13, wherein said normal cells are normal T lymphocytes.

15. The method as recited in claim 14, wherein said activity is the T lymphocyte proliferation.

16. The method as recited in claim 13, wherein said normal lymphocytes are normal B lymphocyte.

17. The method as recited in any one of claims 12 to 16, wherein said activity is the production of antibodies.

18. A method as recited in claim 12, wherein said cells are neoplastic lymphocytes.

19. The method as recited in claim 18, wherein said neoplastic lymphocytes are neoplastic T lymphocytes.

20. The method as recited in claim 19, wherein said neoplastic T lymphocytes are Jurkat cells.

21. The method as recited in any one of claims 12 to 19, wherein said activity is induced by organ or tissue transplant.

22. The method as recited in any one of claims 12 to 20, wherein said activity is induced by an allergen.

23. The method as recited in claim of claim 12 to 22, wherein said activity is induced in autoimmune diseases.

24. A method as recited in any one of claims 12 to 23, wherein said NTPDase inhibitor is selected from the group consisting of BGO 136, erythrosin B, nucleotide or nucleotide derivative including AMP, 8 Bus-AMP and 8 Bus-ATP, and analogues thereof.

25. The method as recited in any one of claims 12 to 23, wherein said inhibitor is BGO136 or a BGO136 analogue.

26. The method as recited in any one of claims 12 to 23, wherein said inhibitor is AMP or an AMP analogue.

5 27. The method as recited in any one of claims 12 to 23, wherein said inhibitor is 8 Bus-AMP.

28. The method as recited in any one of claims 12 to 23, wherein said inhibitor is ATP or an ATP analogue.

10 29. The method as recited in any one of claims 12 to 23, wherein said inhibitor is 8 Bus-ATP.

30. The method as recited in any one of claims 12 to 23, wherein said inhibitor is erythrosine B or an erythrosin B analogue.

15 31. A method to prevent or reduce the risk of rejection of transplanted tissue or organ, comprising administering to said animal an effective amount of NTPDase inhibitor.

32. A method as recited in claim 31, wherein said NTPDase inhibitor is selected from the group consisting of BGO 136, erythrosin B, nucleotide or nucleotide derivative including AMP, 8 Bus-AMP and 8 Bus-ATP, and analogues thereof.

20 33. The method as recited in claim 32, wherein said inhibitor is BGO136 or a BGO136 analogue.

34. The method as recited in claim 32, wherein said inhibitor is AMP or an AMP analogue.

25 35. The method as recited in claim 32, wherein said inhibitor is 8 Bus-AMP.

36. The method as recited in claim 32, wherein said inhibitor is ATP or an ATP analogue.

37. The method as recited in claim 32, wherein said inhibitor is 8 Bus-ATP.

30 38. The method as recited in claim 32, wherein said inhibitor is erythrosine B or an erythrosin B analogue.

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39. The method as recited in claim 32, wherein said inhibitor is BGO 136.

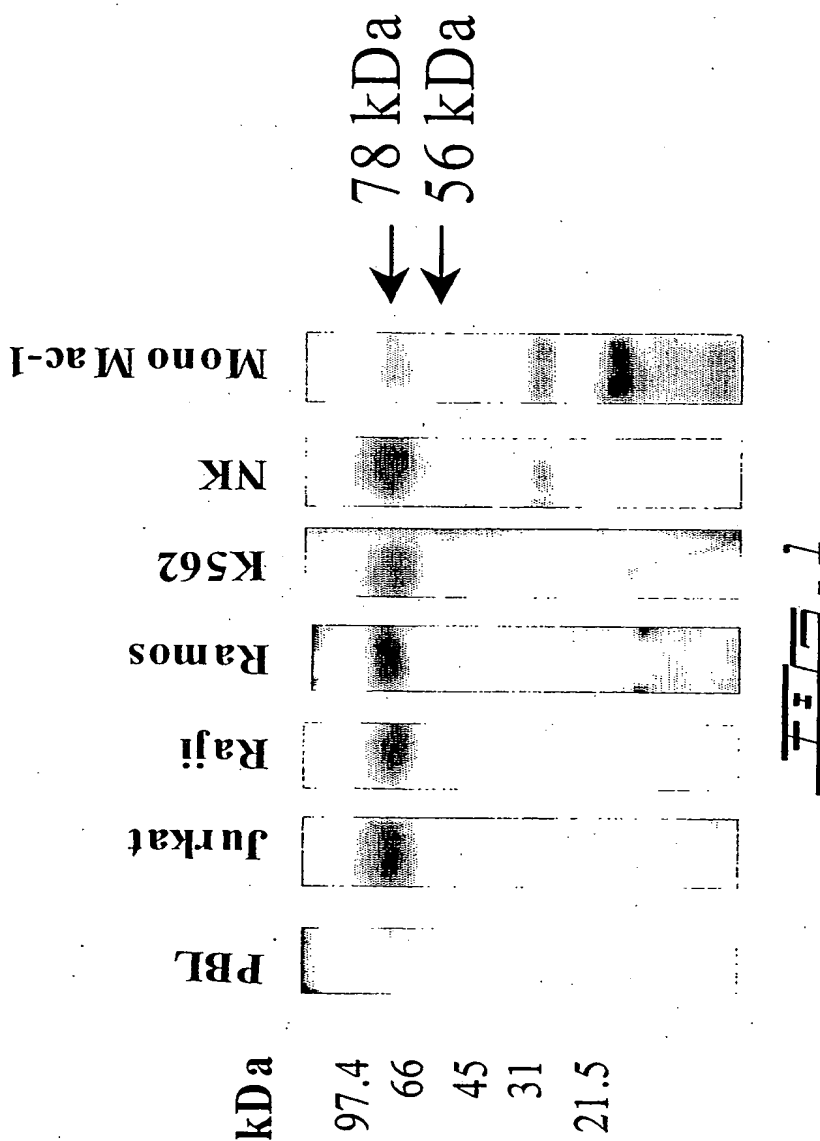
40. Composition for use as an immunosuppressive agent in graft transplant comprising an effective amount of BGO 136 or BGO 136 analogue in a
5 pharmaceutically acceptable carrier.

41. Use of a BGO 136 or BGO 136 analogue in the making of a medicament for use as an immunosuppressive agent in graft transplant.

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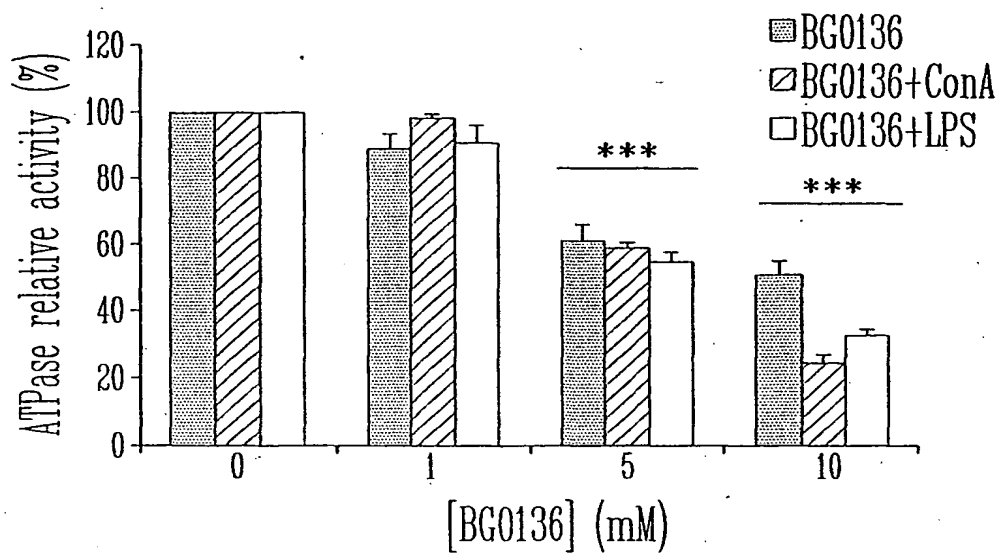
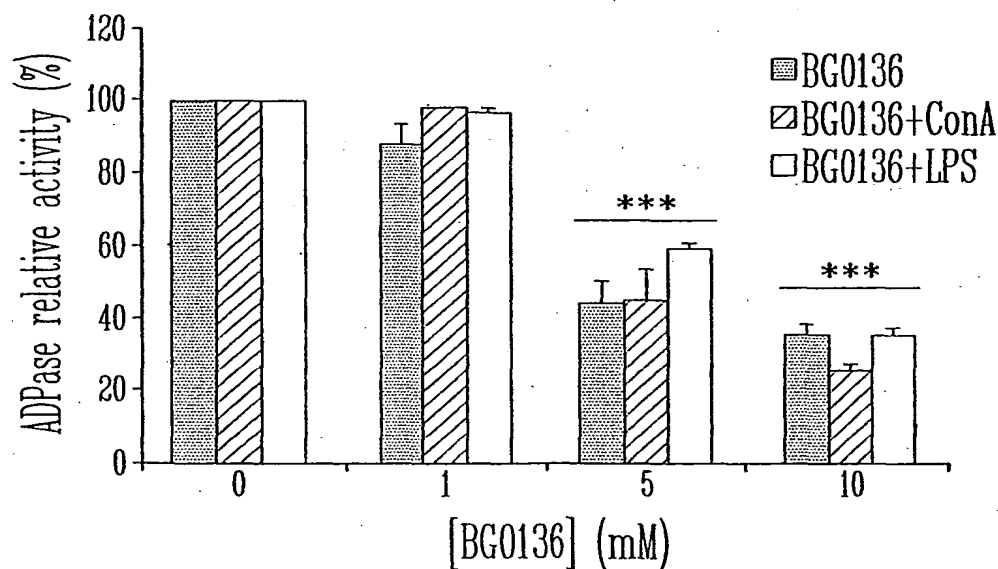


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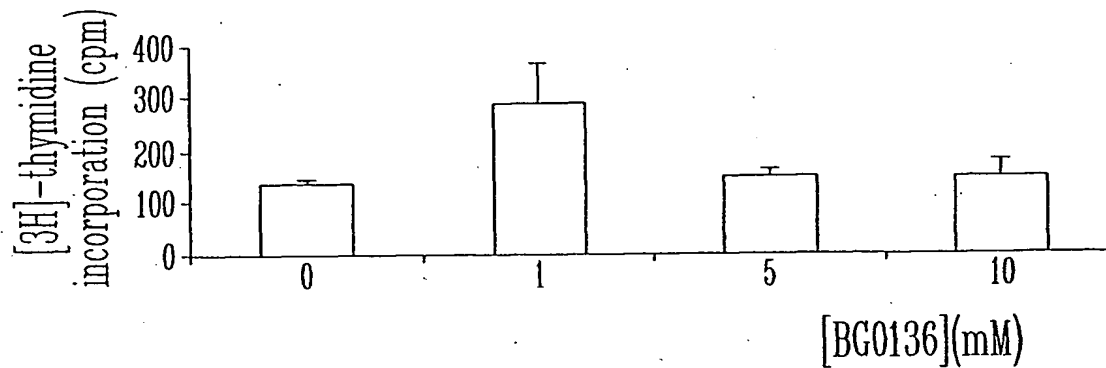
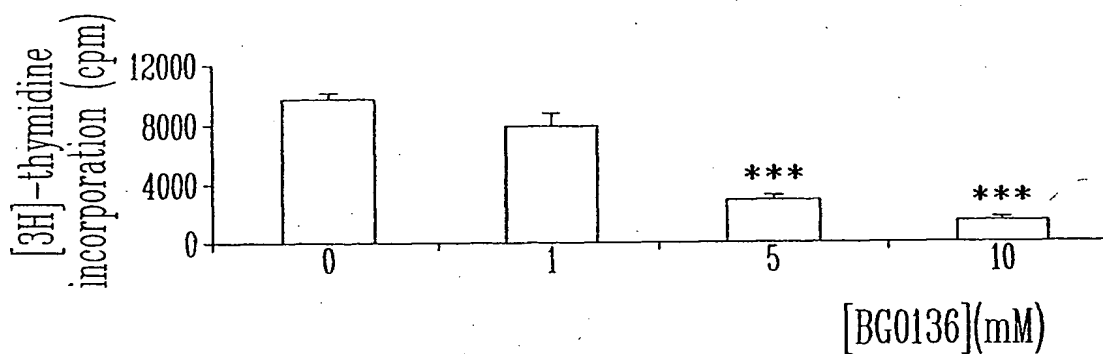
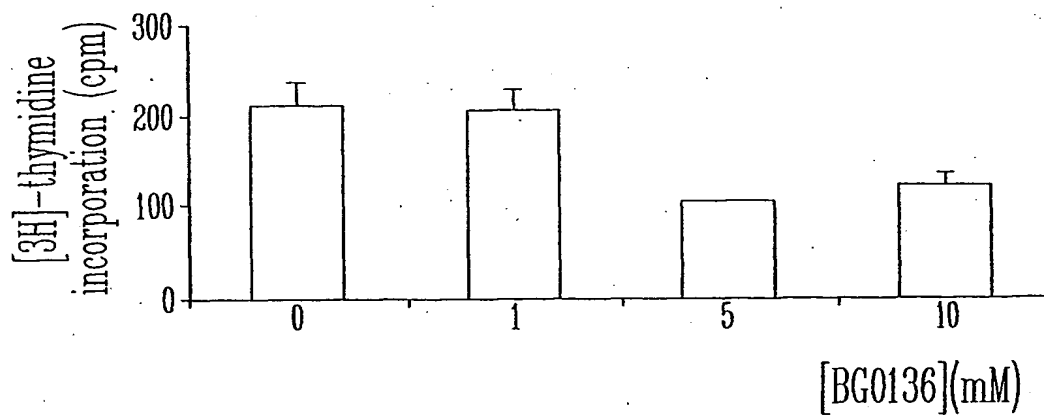
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FIG. 2AFIG. 2B

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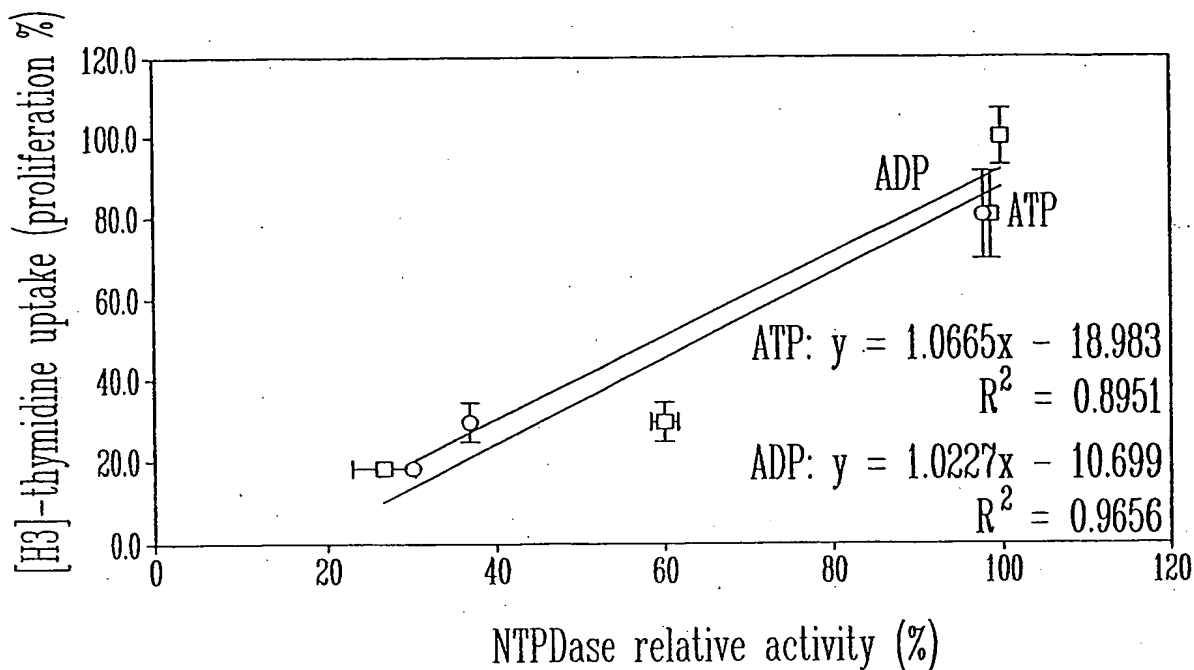
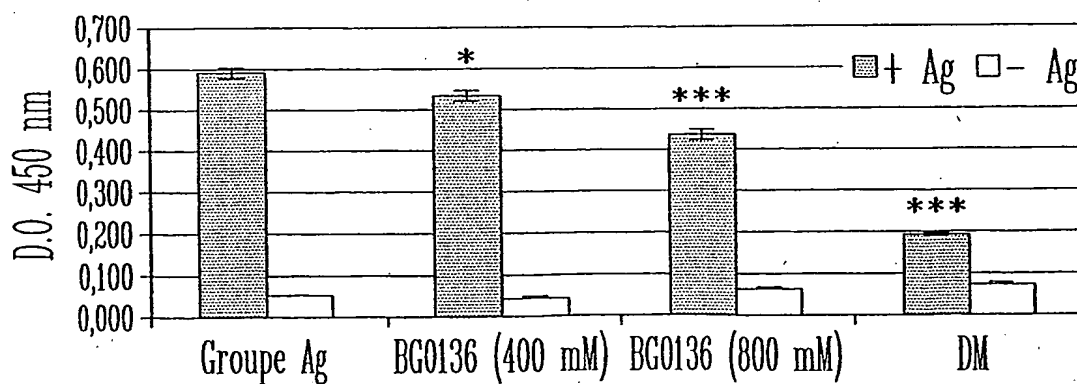
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FIG. 3AFIG. 3BFIG. 3C

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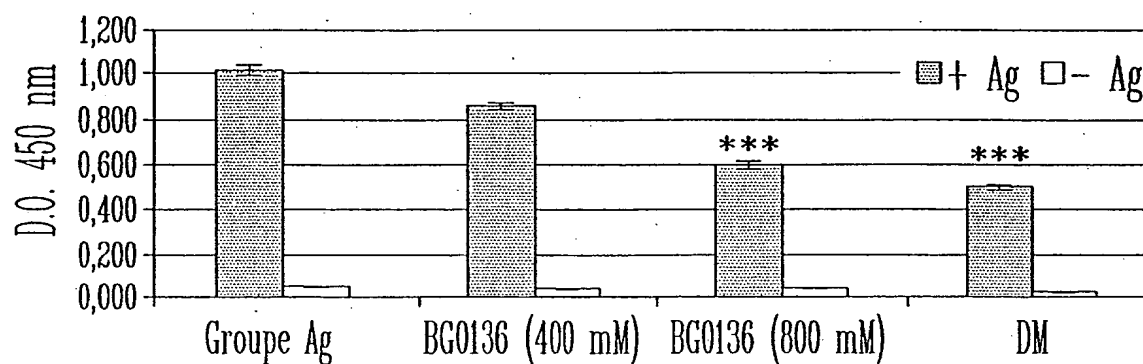
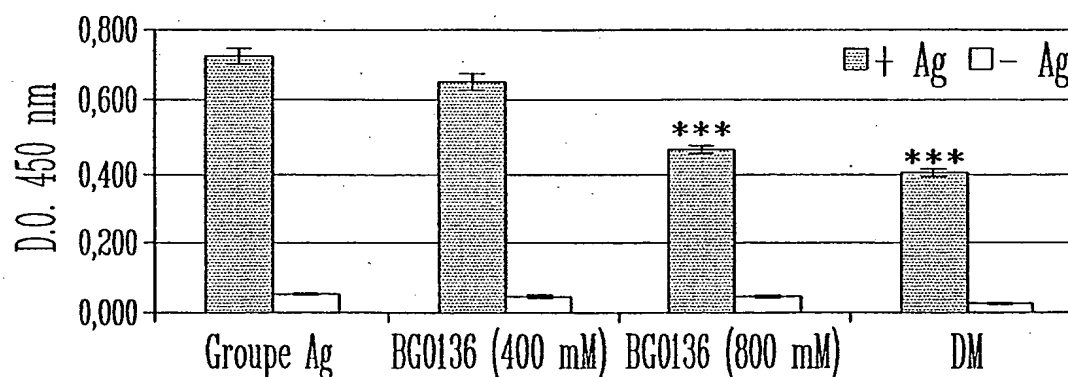
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FIG - 4FIG - 5A

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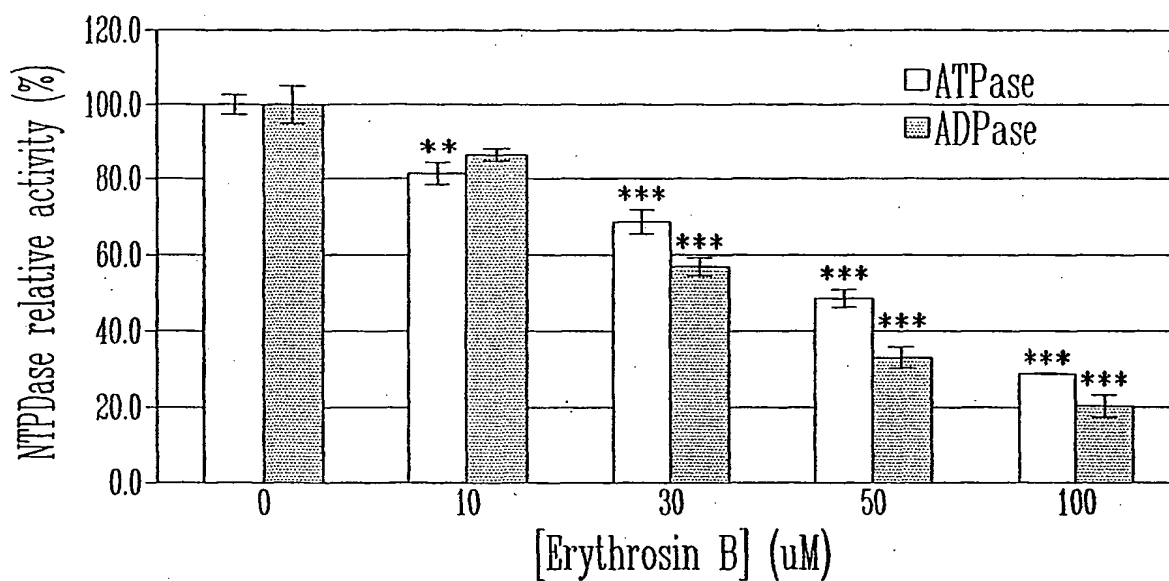
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FIG. 5BFIG. 5C

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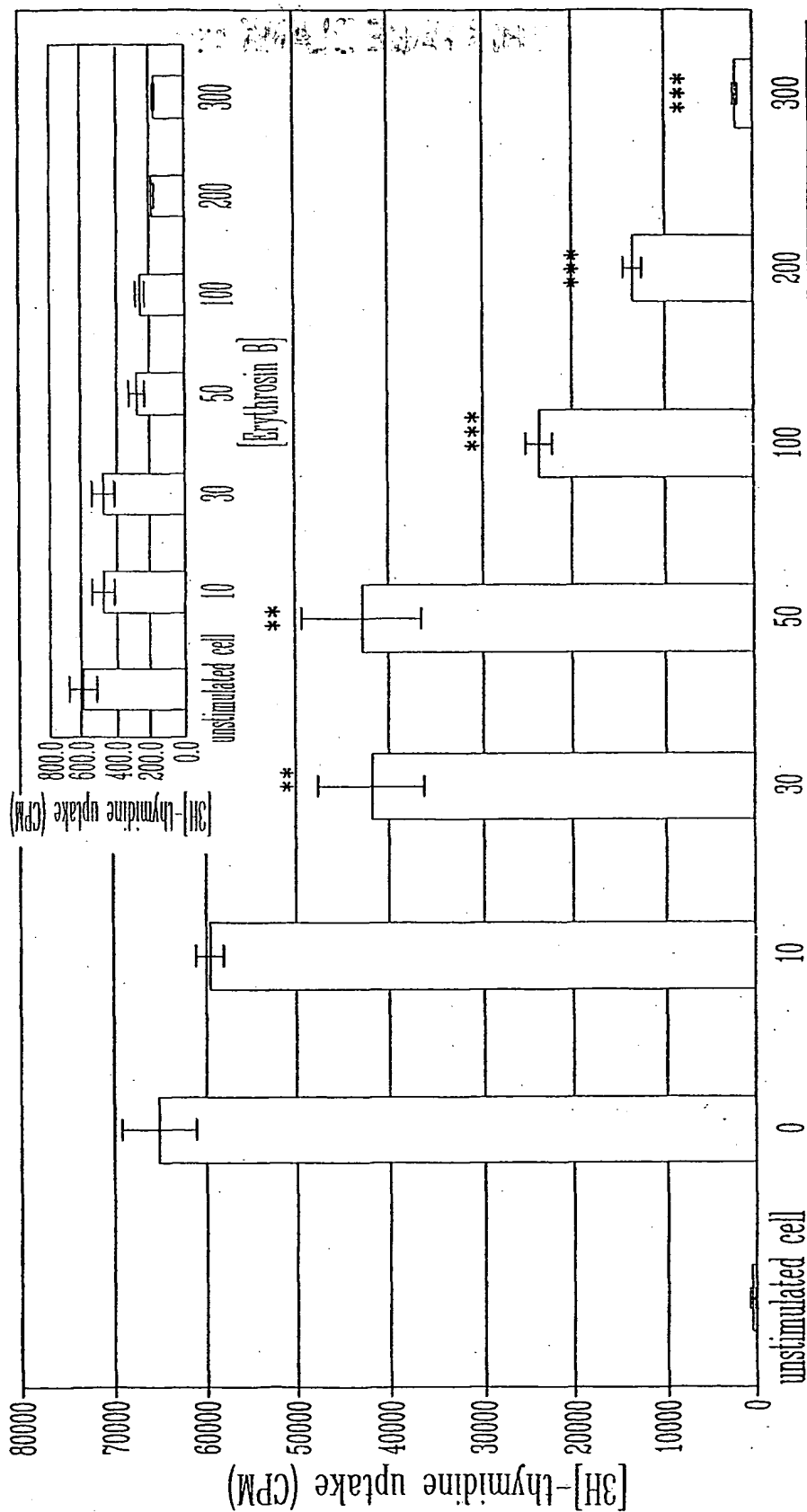
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FIG. 6

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Stimulated cells by Con A +/- [Erythrosin B] (μM)

Fig. 7

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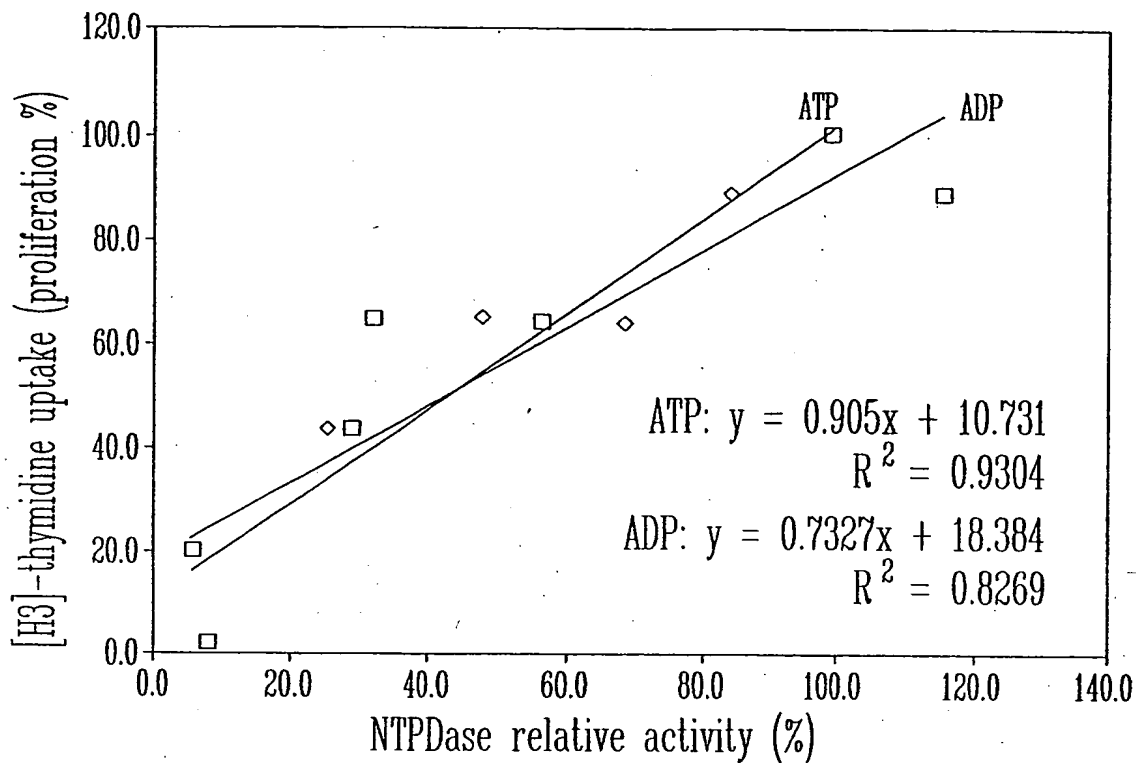


FIG. 8

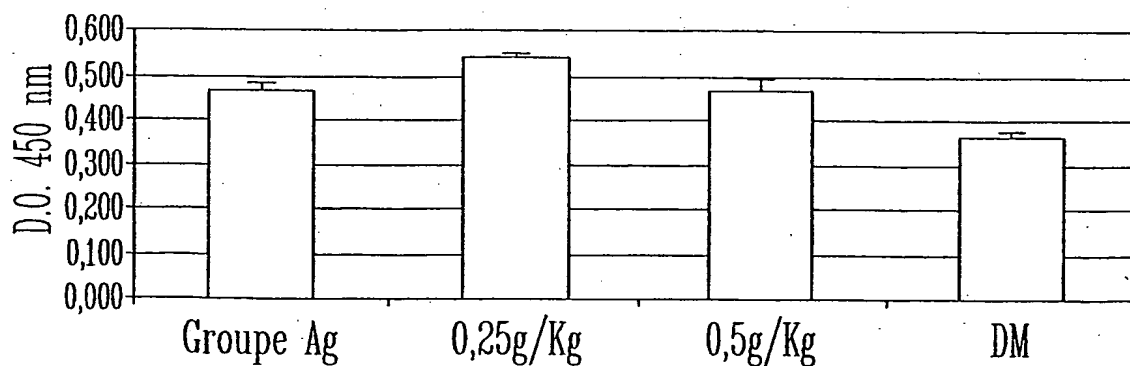


FIG. 9A

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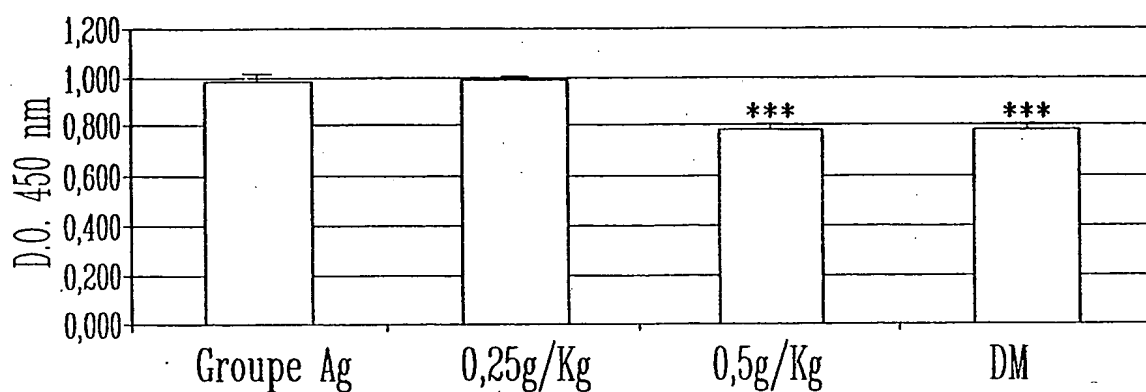


FIG. 9B

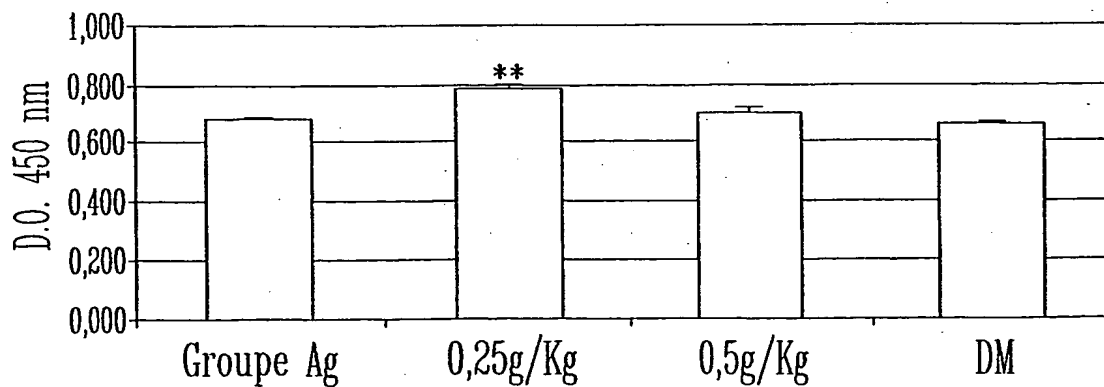
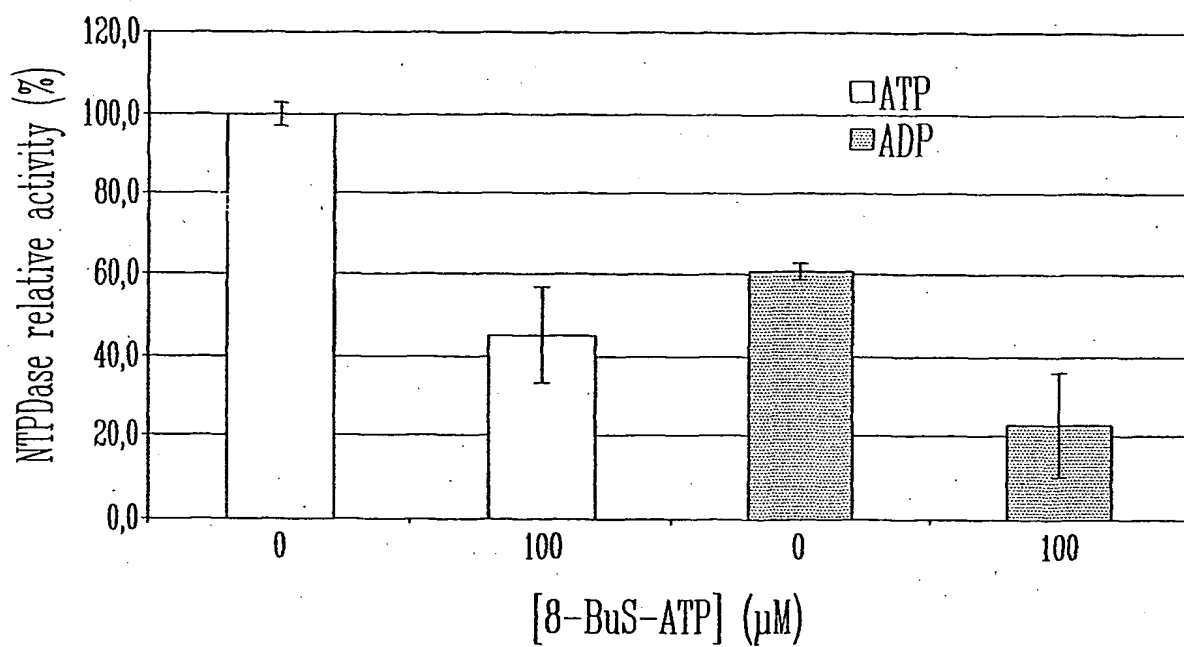


FIG. 9C

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FIG. 10

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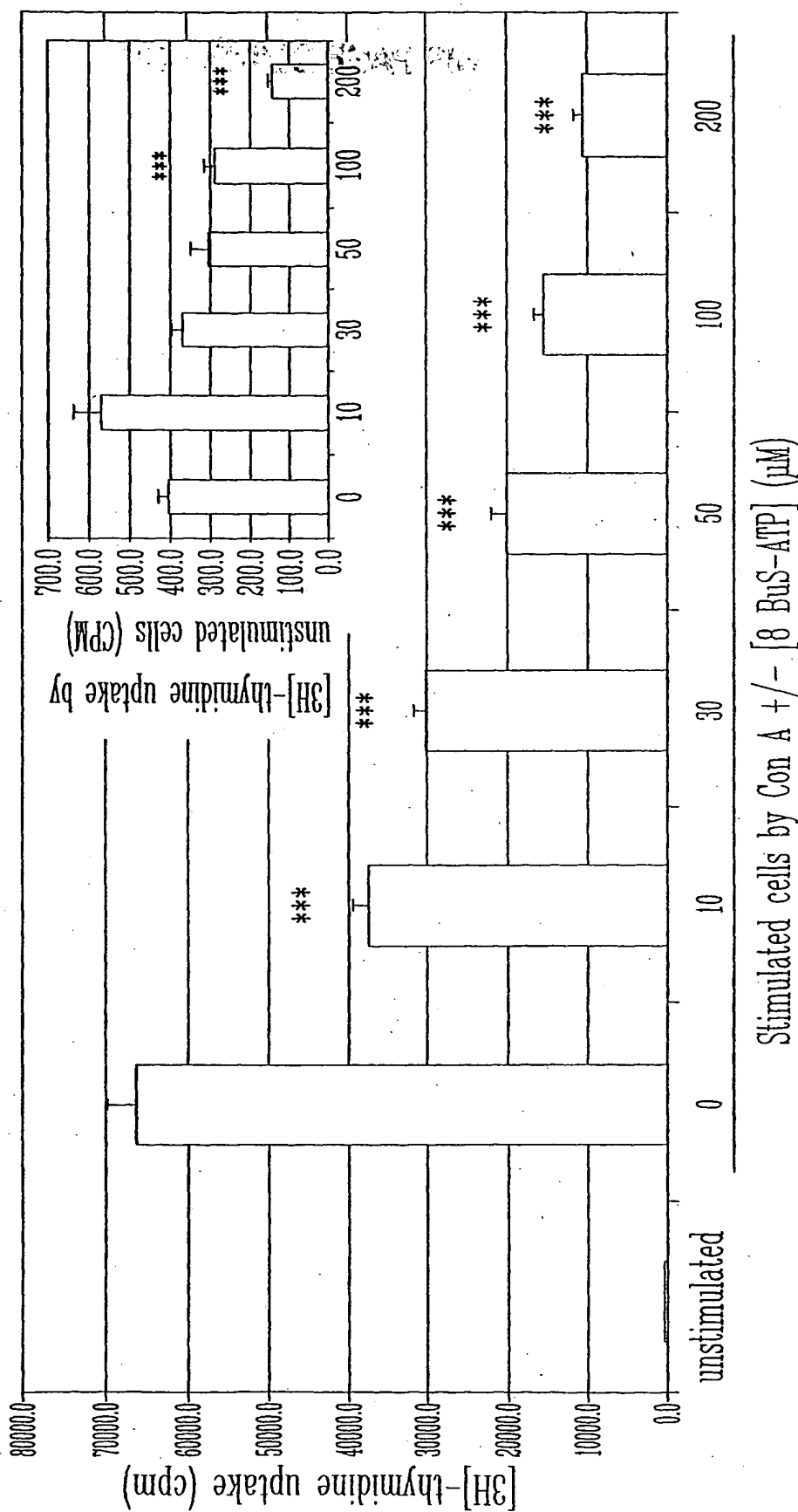


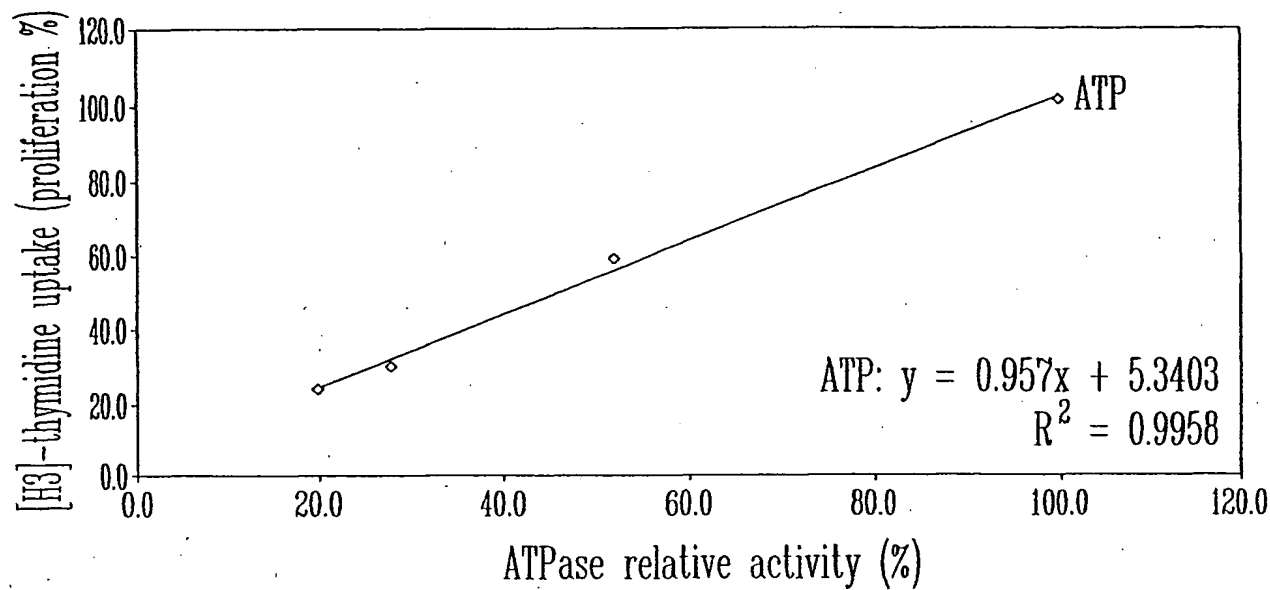
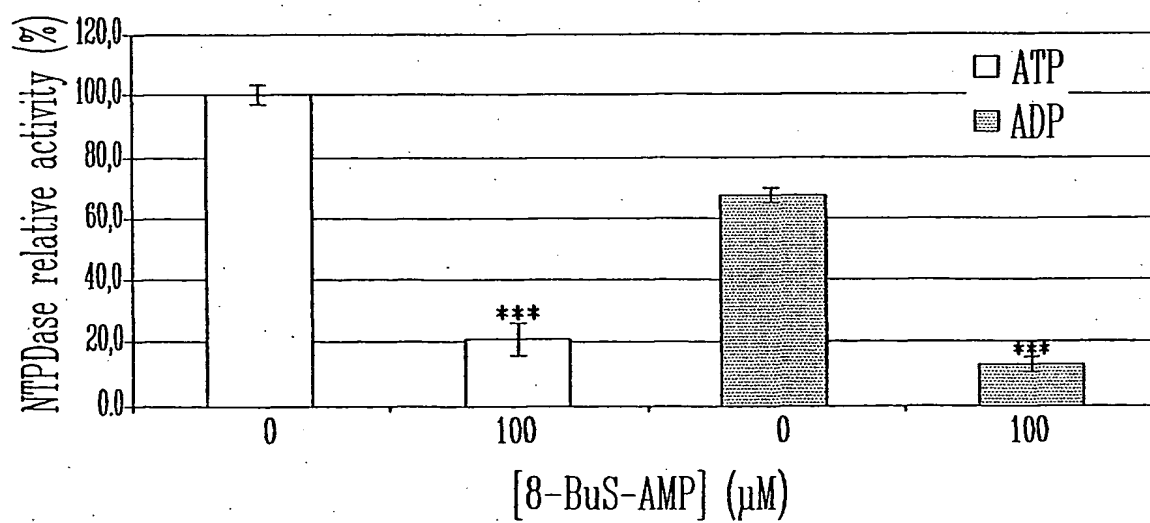
FIG. 11

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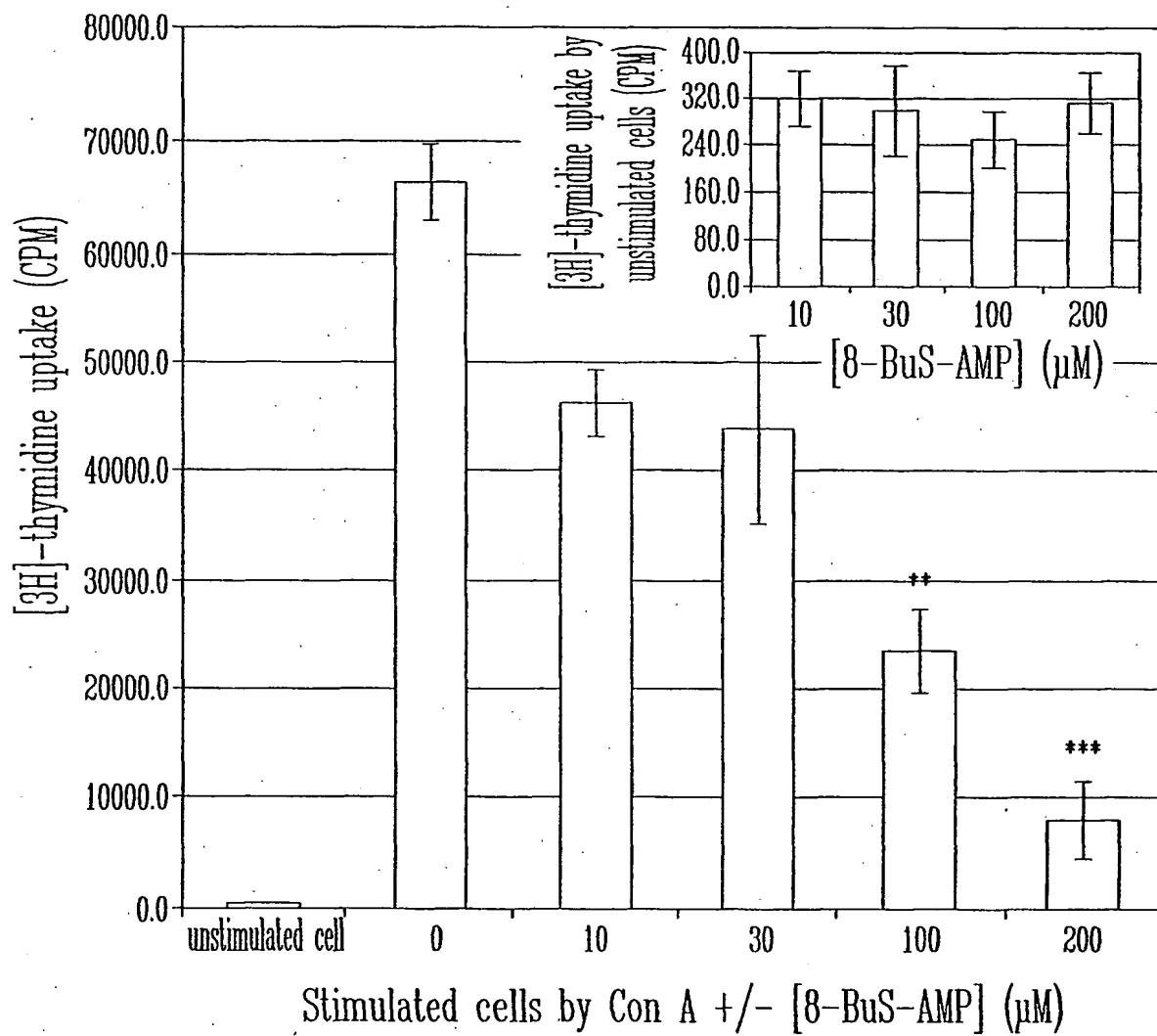
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FIG. 12FIG. 13

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FIG. 14

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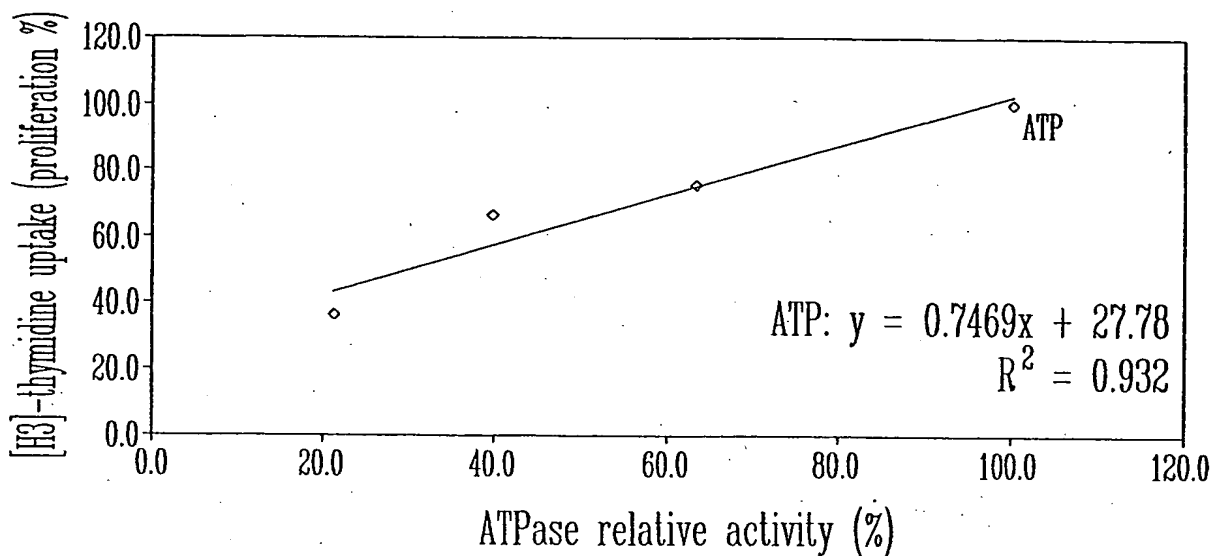


FIG. 15

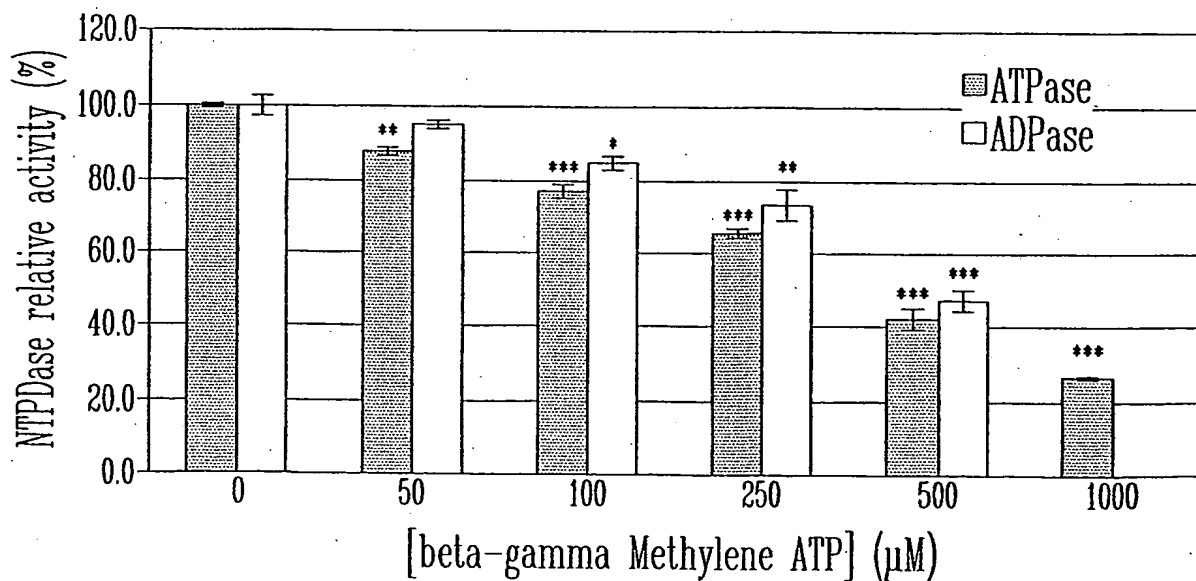
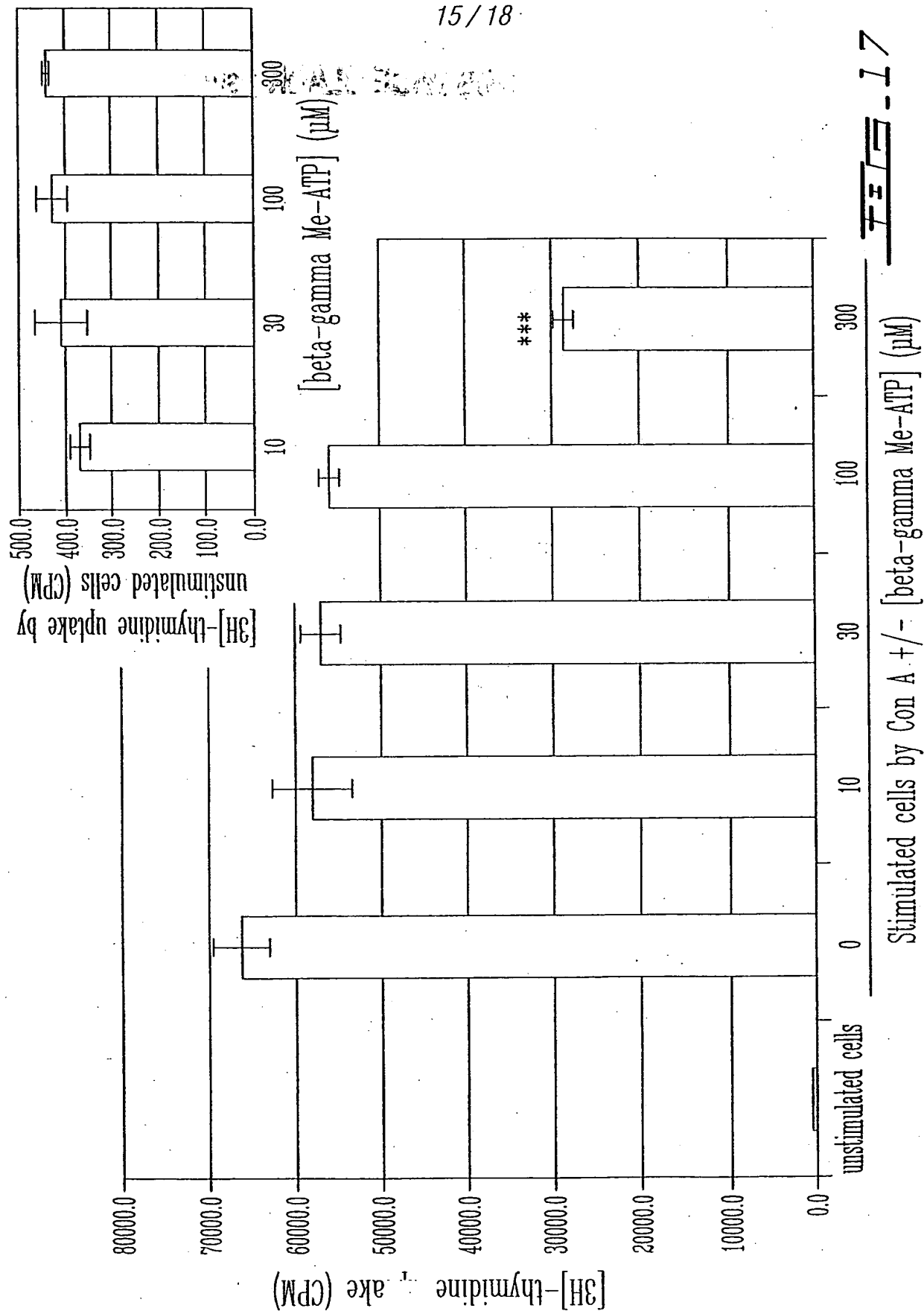


FIG. 16

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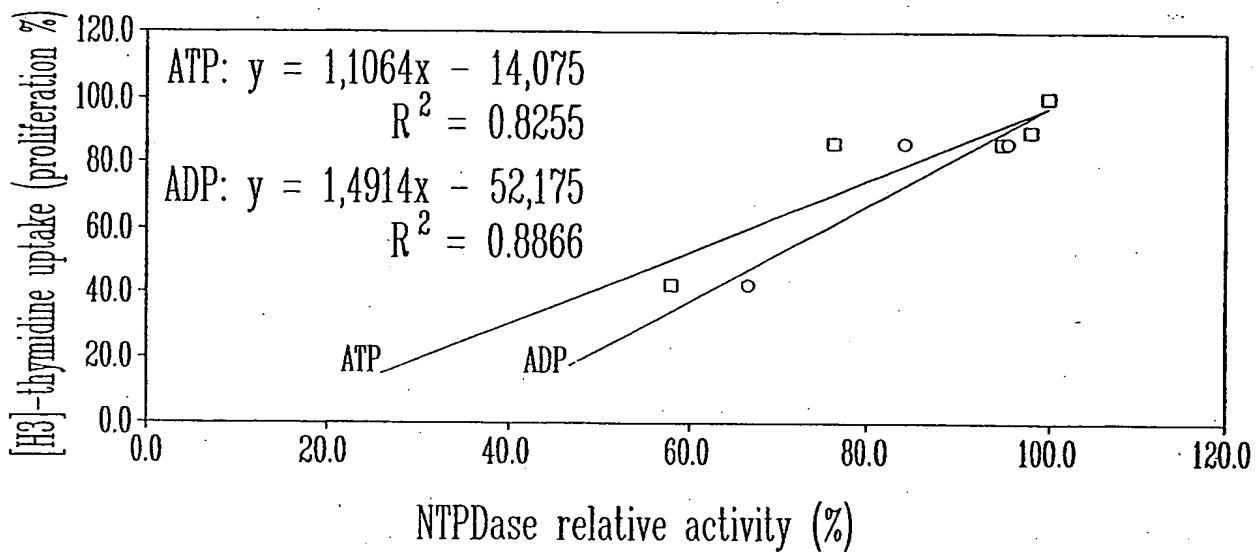
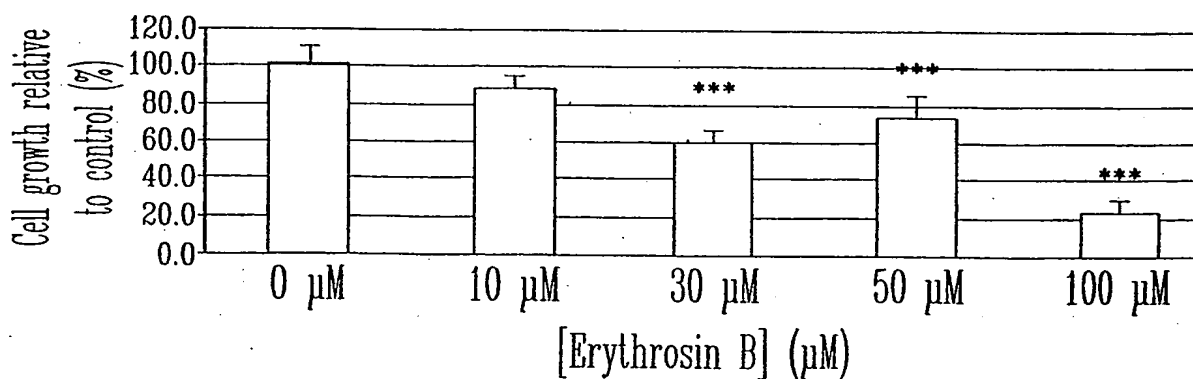
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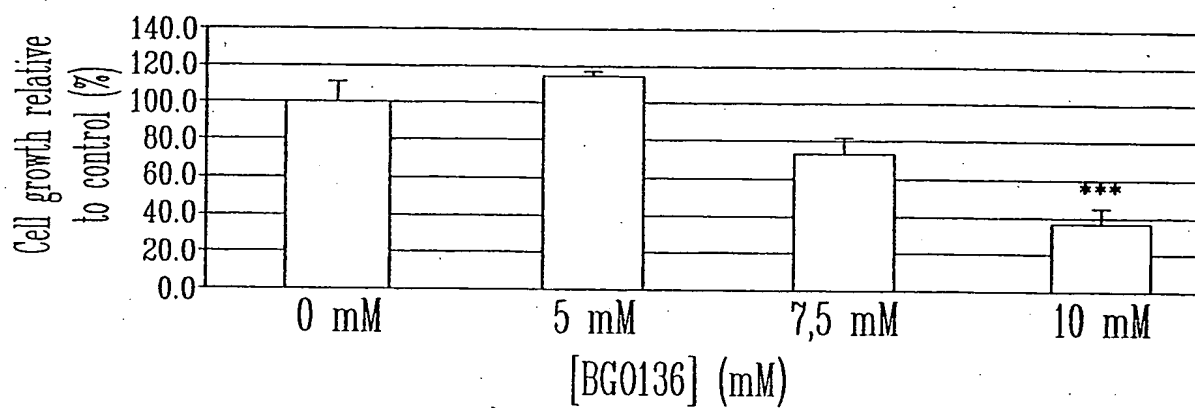
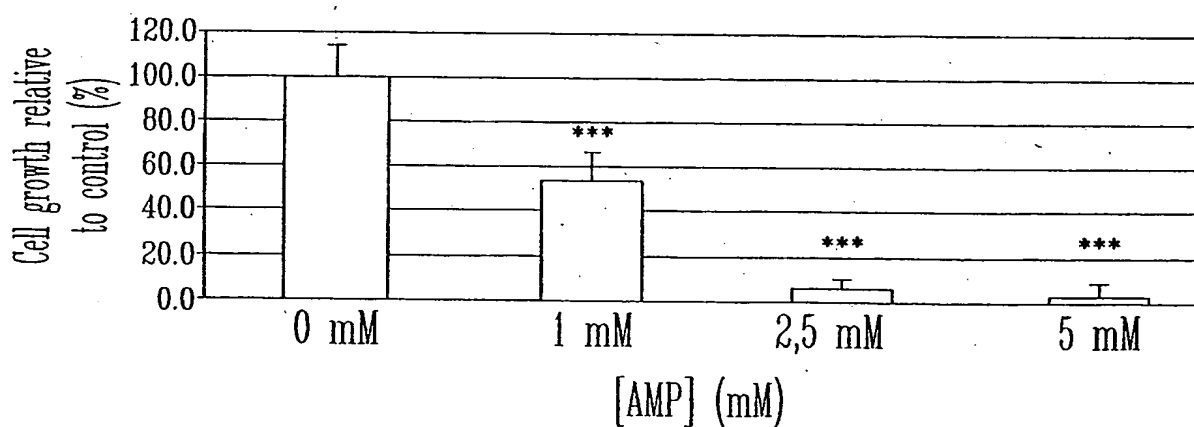
FIG. 1BFIG. 1BA

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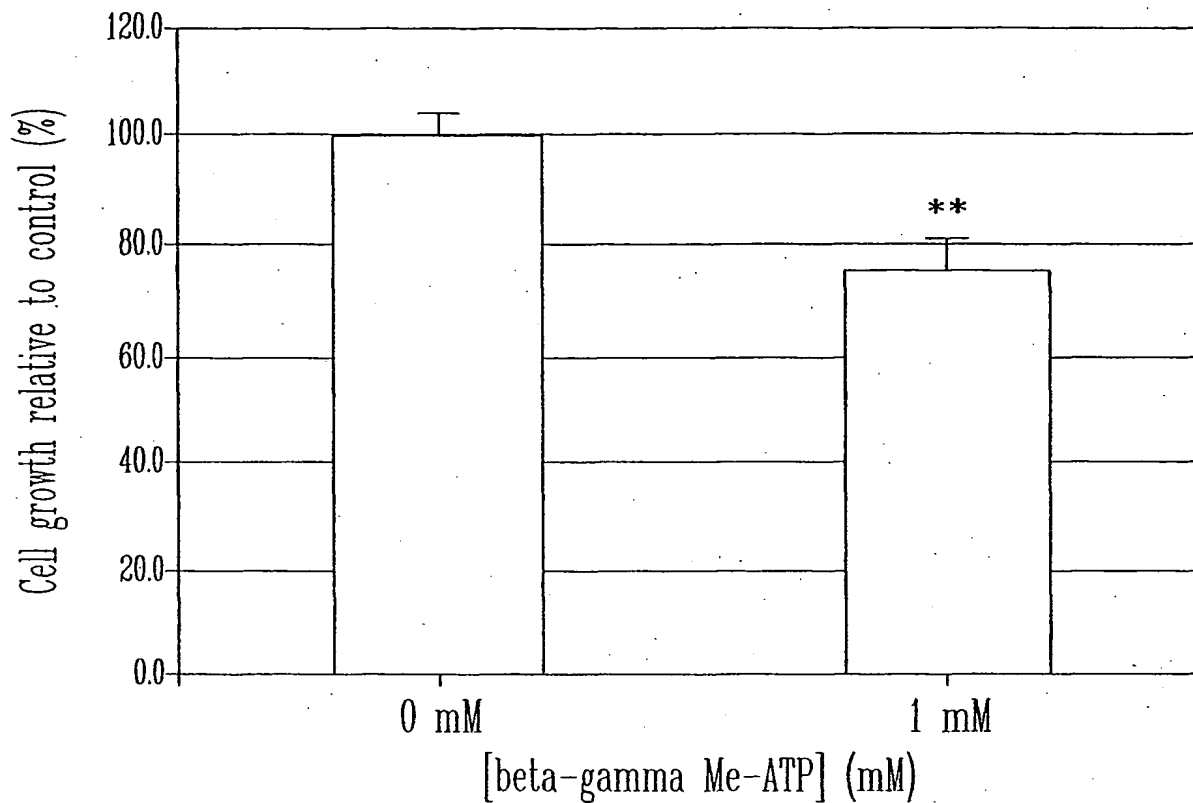
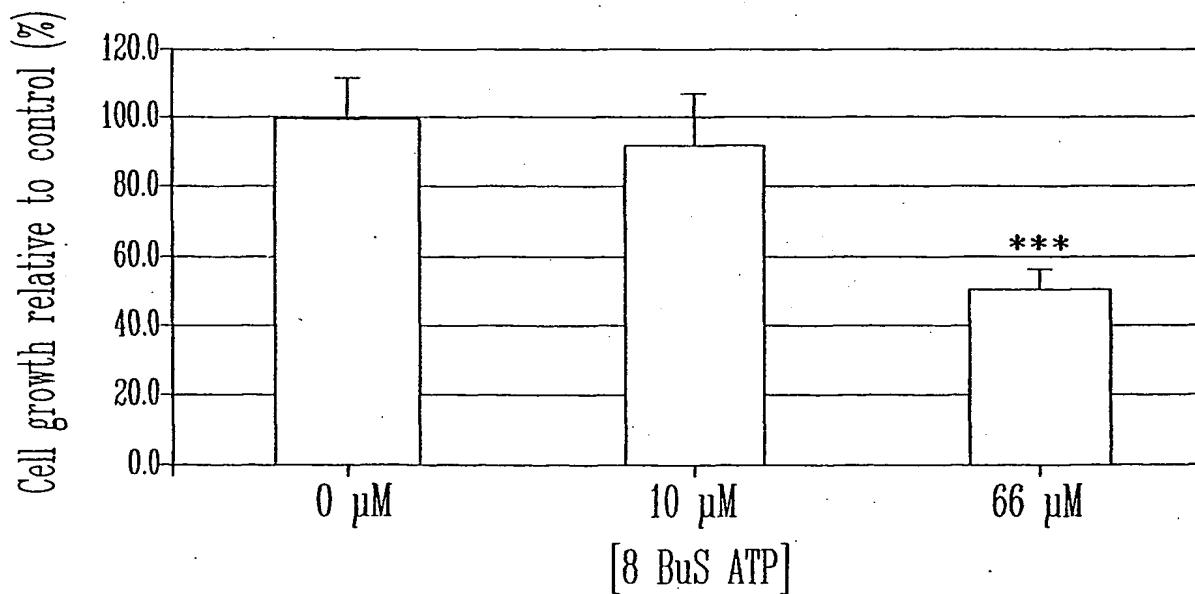
FIG. 19BFIG. 19C

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FIG. 190FIG. 191

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INTERNATIONAL SEARCH REPORT

 Application No
 PCT/CA 03/00583

 A. CLASSIFICATION OF SUBJECT MATTER
 IPC 7 C12Q1/42

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

 Minimum documentation searched (classification system followed by classification symbols)
 IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MIZUMOTO NORIKATSU ET AL: "CD39 is the dominant Langerhans cell-associated ecto-NTPDase: Modulatory roles in inflammation and immune responsiveness." NATURE MEDICINE, vol. 8, no. 4, April 2002 (2002-04), pages 358-365, XP002251343 April, 2002 ISSN: 1078-8956 p.360, left-hand column, 1. 3-5 and "discussion" section	1, 12, 22-30
X	& NATURE MEDICINE, 'Online! Retrieved from the Internet: <URL:http://www.nature.com/dynasearch/app/Dynasearch.taf?_action=search&sp-n=1&sp_k=&search_fulltext=&sp-p=Any&sp-w=Exact&search_volume=8&search_startPage=358> 'retrieved on 2003-08-14! --- -/--	1, 12, 22-30

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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- *A* document defining the general state of the art which is not considered to be of particular relevance
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Date of the actual completion of the international search

18 August 2003

Date of mailing of the International search report

01/09/2003

Name and mailing address of the ISA

 European Patent Office, P.B. 5818 Patentlaan 2
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 Fax: (+31-70) 340-3016

Authorized officer

Jacques, P

INTERNATIONAL SEARCH REPORT

Intern. application No.
PCT/CA 03/00583

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 12-39 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GRANDSTEIN R.D.: "The skinny on CD39 in immunity and inflammation" NATURE MEDICINE, vol. 8, no. 4, April 2002 (2002-04), pages 336-338, XP002251344 the whole document ---	1,12, 22-30
P,X	WO 02 071062 A (TAKASHIMA AKIRA ;UNIV TEXAS (US); KUMAMOTO TADASHI (US); MIZUMOTO) 12 September 2002 (2002-09-12) page 19, line 5 - line 6 page 19, line 32 - line 36 claim 53 claim 73 claim 81 ---	1,12, 22-30
A	GENDRON FERNAND-PIERRE ET AL: "Novel inhibitors of nucleoside triphosphate diphosphohydrolases: Chemical synthesis and biochemical and pharmacological characterizations." JOURNAL OF MEDICINAL CHEMISTRY, vol. 43, no. 11, 1 June 2000 (2000-06-01), pages 2239-2247, XP002251346 ISSN: 0022-2623 the whole document ---	1-41
A	CA 2 311 084 A (UNIV SHERBROOKE ;UNIV BAR ILAN (IL)) 9 December 2001 (2001-12-09) the whole document -----	1-41

INTERNATIONAL SEARCH REPORT

Information on patent family members

Application No

PCT/CA 03/00583

Patent document cited in search report		Publication date		Patent family member(s)	Publication date
WO 02071062	A	12-09-2002	US	2003087247 A1	08-05-2003
			WO	02071062 A2	12-09-2002
CA 2311084	A	09-12-2001	CA	2311084 A1	09-12-2001

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